STUDIES ON THE CHEMISTRY OF PROTOZOA

- by -

Andrew Ronald Archibald, B.Sc.

Thesis presented for the degree of Doctor of Philosophy.

University of Edinburgh

August, 1958.

CONTENTS

Index of Figures

Nomenclature

For convenience, the following abbreviations and terms have been used:-

specific optical rotation; all values recorded were measured at approximately 18°C.

C.L., average chain length, denotes the number of monomer residues per non-reducing terminal residue of a polysaccharide.

D.P., average degree of polymerisation, refers to the number of monomer residues per molecule of a polysaccharide.

M and N denote molar and normal concentration of solution, respectively.

R.P., reducing power, refers to the number of monomer residues per apparent reducing group, determined as equivalents of standard sugars.

$R_{g.}$ value, refers to the distance travelled by the compound in a given chromatographic solvent relative to that travelled by glucose.

SECTION I

GENERAL INTRODUCTION.

Protozoa, the most primitive members of the animal kingdom, occur in great variety in fresh and salt water, in damp soil and, as parasites, in other animals. Protozoa may be simply defined as animal protists. The term "protist" was coined by Haeckel (1) to designate "organisms composed of a single cell which are capable within the limits of that cell of performing all the functions necessary for their continuance in life and also for their propagation". The formal description of the term "animal" as "a living organism having sensation and power of voluntary movement" presents some difficulty however. Furthermore, the commonly accepted criterion that animals are phagotrophic organisms, i.e. are able to ingest particulate food, can not be applied strictly to all protozoa. Certain protozoa are parasitic and are not phagotrophic, although by their metabolism and relationship to other organisms they are most suitably classed as animals. Similarly, it is unsatisfactory to consider the possession of chlorophyll by an organism as conclusive evidence that the organism is in fact a plant. Many species are known, e.g. Chrysamoeba, which contain chlorophyll and yet, in other respects, show typical animal properties; for example, they are phagotrophic. Hutner and Provasoli (2) suggest that all permanently colourless forms, as well as all nucleated phagotrophic protists, should be considered members of the phylum Protozoa.

AMOEBA PROTEUS

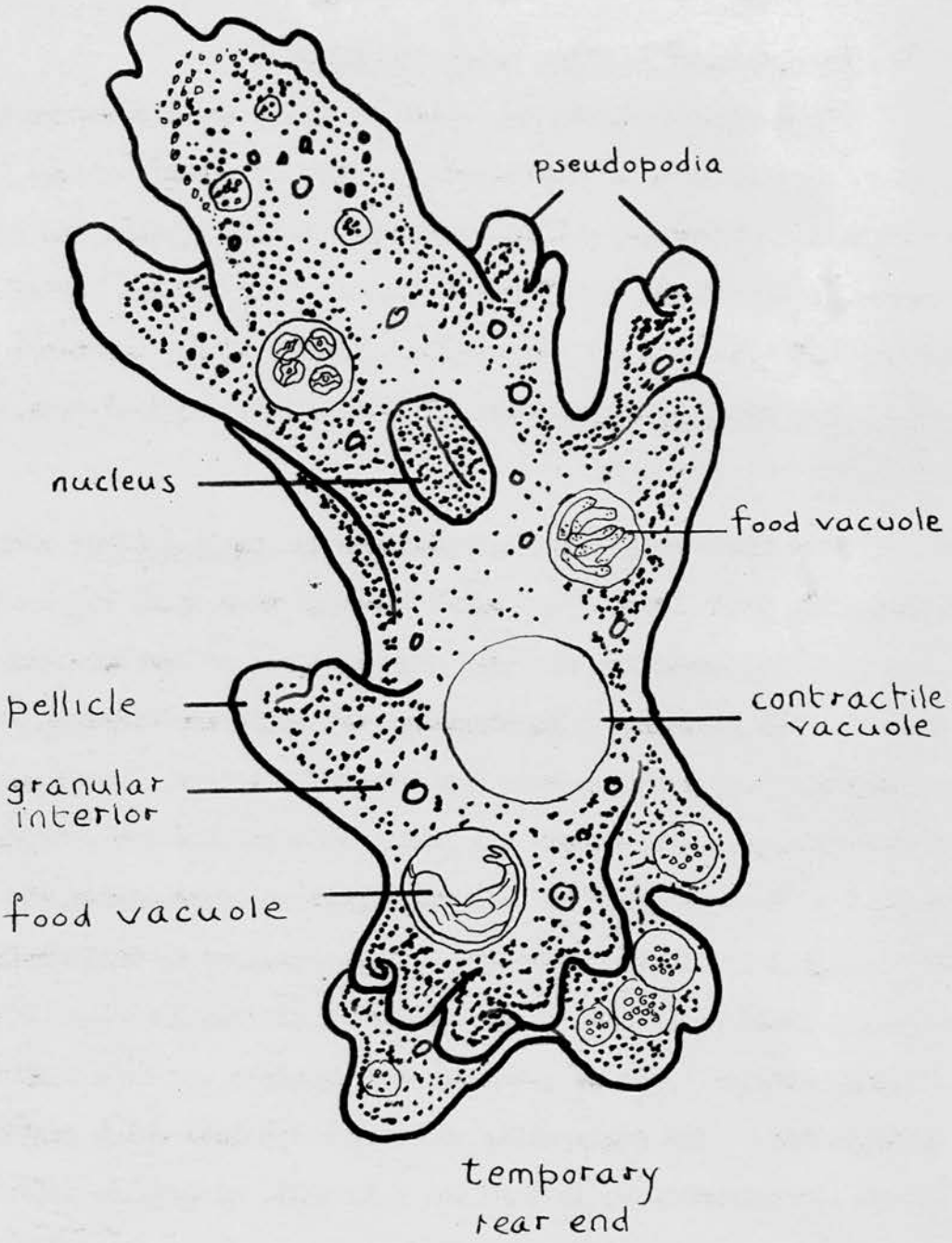


Fig. 1

I The Classification of Protozoa

Very many species of protozoa have been described (4) (5) (6). These may be conveniently divided, according to their chief method of locomotion, into four classes:- the amoeboid protozoa, the flagellates, the spore-forming protozoa and the ciliates.

(a) The Amoeboid Protozoa (class SARCODINA):-

This class includes all those organisms which move about and capture food by means of pseudopodia. Many of these protozoa live free in fresh and salt water and in damp soil; others inhabit the interior of animals, particularly the digestive tract. Many of the latter are harmless to the host animal, but a few species attack the host and cause disease, e.g. Endamoeba histolytica which, in man, causes tropical dysentery and abscess of the liver, (7).

The most common amoeboid protozoa is Amoeba proteus which lives in fresh water ponds (Fig. 1). Apart from the mechanisms responsible for locomotion and ingestion of food, many features of the amoeboid cell are common to all protozoa. For example, the cells are composed of gelatinous protoplasm which contains many granules and droplets. This protoplasm is differentiated, as in almost all cells, into nucleus and cytoplasm. The nucleus is that part of the cell which governs reproduction and the constructive phases of metabolism. The cytoplasm is distinguishable into a clear, outer layer (pellicle) and a more or less granular interior which contains various types of granules, fat droplets and food bodies in process of digestion. The contractile vacuoles - droplets which contain water - effect the elimination, through the cell wall, of surplus water. The cell wall in amoebas is a delicate semi-permeable membrane formed by the surface of the cytoplasm. An amoeba has no fixed shape, and any one point on the

surface of the cell may flow out as a blunt projection or pseudopodium. By the passage into it of some of the mass of the amoeba, this pseudopodium continues to advance for some time. The cell is capable of producing many such pseudopodia by virtue of which it moves and ingests particulate food.

(b) The Flagellates (class FLAGELLATA):-

These are protozoa which have a cell of definite shape and one or more long filamentous protoplasmic extensions (flagella) by means of which they are capable of active movement. Flagellates may be further divided into two groups:- the animal-like and the plant-like (i.e. algal) groups. Examples of the animal-like group are the collar flagellates, e.g. Codosiga, which live attached to the substratum by means of a stalk. By beating their flagella, these organisms direct a current of water on to the cell which then engulfs any food particles which strike the cell wall.

Many parasitic forms are included among the animal-like flagellates, the best known of which are the trypanosomes which cause African sleeping sickness in man.

Euglena is a common example of a plant-like flagellate in that it possesses photosynthetic bodies (green chloroplasts) and is not phagotrophic.

Many flagellates appear to be intermediate between the true protozoa and the algae. One such flagellate is Chrysamoeba which has a yellow-green photosynthetic pigment. This form sometimes loses the flagellum; it then moves about and ingests solid food by means of pseudopodia.

Plant-like flagellates are the most primitive group of protozoa and are generally considered to occupy a systematic position at the intersections of plant and animal lines of descent.

(c) The Spore Formers (class SPOROZOA):-

These have no special mode of locomotion and are all endo-parasites, some of which, e.g. Plasmodium falciparum, cause diseases such as malaria in man. They are characterised by their method of reproduction, and reproduce by 'spores' in the formation of which the nucleus of a protozoon divides several times; a little cytoplasm then gathers round each nucleus and the protozoon falls apart into a number of offspring corresponding to the number of nuclei formed.

(d) The Ciliates (class CILIATA):-

These are distinguished from other protozoa by the possession of numerous cilia or 'hairs', the movement of which provides the method of locomotion. Ciliates are among the most highly evolved protozoa. Some ciliates, e.g. Stylonychia mytilus, may be parasitised by other protozoa, e.g. Acinetans.

Ciliates, flagellates and amoeboid protozoa usually reproduce by 'budding' which involves division of the nucleus into two similar parts. The cell then splits to yield two smaller cells which are capable of growth and further reproduction. Natural death does not therefore occur in protozoa. Many species, however, are capable of sexual reproduction. In some of these species the two fusing cells are alike; in others, sexual evolution is complete and fully differentiated male gametes, or sperms, and female gametes, or eggs, are formed.

The present work has been mainly concerned with flagellates and ciliates; a list of the species discussed is given in Table 1.

TABLE I

FLAGELLATA	CILIATA
(a) Plant-like	Cycloposthium
Chilomonas paramecium	Holotrich ciliates
Euglena	Tetrahymena pyriformis
Ochromonas malhamensis	
Polytoma	
Polytomella coeca	
(b) Animal-like	
Trichomonas foetus	
Trichomonas gallinae	

II The Nutrition of Protozoa

Many protozoa do not possess a mouth aperture (cytostome) and are unable to produce pseudopodia. They are therefore able to absorb only dissolved food, mineral or organic, and are known as compulsory osmotrophs. Those protozoa which feed either on particles or on dissolved material are known as facultative phagotrophs or facultative osmotrophs, and those which feed only on particles are known as compulsory phagotrophs.

Many of the flagellates possessing photosynthetic pigments are strict osmotrophs. Some, however, are able to produce pseudopodia at certain phases of their lives and can then ingest particles. Thus phagotrophy may be combined with photosynthesis. Photosynthetic protozoa (phototrophs) utilise only inorganic substances as hydrogen donors for the photoreduction of carbon dioxide; all known non-photosynthetic protozoa (chemotrophs), on the other hand, require an organic energy source and can not grow in a purely inorganic medium.

III The Biochemistry of Protozoa

Compared with present knowledge of the biochemistry of bacteria, relatively little is known about the biochemistry of protozoa. One of the many reasons for this is the difficulty in obtaining bacteria-free cultures of protozoa for experimental studies. Lwoff has made notable contributions to the preparation of such pure cultures. Biochemical investigations are also hindered by the fact that many protozoa grow very slowly and do not develop abundantly, (3).

Since protozoa are the simplest forms of animal life and include several species which appear to be intermediate between animals and plants, biochemical studies are of very great interest. The medical and economic importance of diseases caused by protozoa has led to the development of several synthetic protozoacidal drugs (7) and to a considerable volume of work on the metabolism of certain protozoa which have been obtained in pure culture. A list of some of the more familiar protozoal diseases, the species which give rise to these diseases, and drugs which are used to combat them, is given in Table 2.

Table 2

PROTOZOAL DISEASES

DISEASE	SPECIES	DRUG
Malaria	*Plasmodium viva P. malariae P. ovale, P. falciparum*	Quinine, paludrine
Syphilis	*Treponema pallidum*	Salvarsan, sulpharsphenamine
Sleeping sickness	*Trypanosoma gambiense*	Atoxyl, tryparsamide, germanin.
Yaws	*Treponema pertenue*	Salvarsan

Table 2
(Contd.)

DISEASE	SPECIES	DRUG
Kala-azar	Leishmania donovani	Tartar emetic, amidines
Amoebic dysentery	Endamoeba histolytica	Emetine
African bovine disease	Trypanosoma congolense, T. vivax.	Antrycide

Relatively few of the many known species cause disease, however, and many intestinal parasites such as Endamoeba coli may occur in considerable numbers in man without harmful effects.

The present work has been mainly concerned with the polysaccharides synthesised by protozoa and a review is here given of previous work done in this field.

(a) The Synthesis of Starch by Protozoa.

Starch, the characteristic reserve polysaccharide of green plants, has been identified by iodine staining in several green flagellates and in their colourless homologues (2). Three such starches, from Polytoma (8), Chilomonas paramecium (9) and Polytomella coeca (10) have been examined chemically and shown to resemble typical plant starch (cf. Section V).

In the plant kingdom starch granules are synthesised only in the plastidic line, in chloroplasts or leucoplasts. The fact that plastids may lose their chlorophyll but continue to synthesise starch suggests that the chloroplasts in pigmented protozoa contain a starch-synthesising enzyme system and are not concerned solely with the photoreduction of carbon dioxide. Some species of Euglena, such as Euglena gracilis, when grown in the dark may lose the ability to synthesise chlorophyll; the resulting colourless

form continues to synthesise starch (2). When Euglena gracilis is grown in the dark and when the chloroplasts are disappearing, some chloroplasts are seen to establish intimate connections with the mitochondrial system. It therefore seems possible that the whole of the chloroplast is not lost and that a colourless leucoplast persists. If Euglena mesnili is treated with streptomycin the chloroplasts are lost. The chloroplast-less flagellate is unable to live in the dark whereas as long as some of the chloroplasts are retained, Euglena mesnili is able to grow in the absence of light. In this instance photosynthesis is not essential for life, whereas chloroplasts are. It therefore appears that some essential enzyme systems are bound to the chloroplasts and that in the starch-synthesising colourless forms these essential enzyme systems continue to exist in remaining leucoplasts.

Lwoff (3) has observed that, of the non-photosynthetic protozoa, starch is synthesised only by those species which have a more or less specialised nutrition. They are unable to utilise sugars and amino acids but can utilise organic acids, particularly the lower fatty acids.

There are three known mechanisms of synthesis of α-1:4 - glucosidic linkages which account for 95% of the linkages in the starch molecule.

(1) by phosphorylase:-

$$n[\alpha\text{- D - glucopyranose - 1 - phosphate}] \rightleftharpoons (\text{glucose})_n + n\,\text{ortho-phosphate}$$

(2) by amylomaltase:-

$$n[4\text{ - }(\alpha\text{- D - glucopyranosido) - D - glucopyranose}] \rightleftharpoons (\text{glucose})_n + n\,\text{glucose}$$

and (3) by amylosucrase:-

$$n[1\text{ - }(\alpha\text{ - D - glucopyranosido) - 2-}\beta\text{-D - fructofuranose}] \rightleftharpoons (\text{glucose})_n + n.\,\text{fructose}$$

A study of protozoal starch synthesis has been made only in Polytomella

coeca (10) (11). This flagellate has been shown to contain a true phosphorylase indicating that starch is synthesised by method (1). This is the most common method of starch synthesis. In starch-synthesising organisms it is probable that glucose - 1 - phosphate is synthesised from triose phosphate and this, in turn, from phosphoenol pyruvic acid. Glucose - 1 - phosphate, fructose - 6 - phosphate, fructose - 1:6 - di-phosphate and phosphoglyceric acid have been detected in Euglena gracilis (2). Carbohydrate synthesis therefore appears to proceed essentially as shown in Scheme I (reactions 2 - 10). Reactions a_1, a_2, b and c are unknown hypothetical reactions leading to the synthesis of phosphoenol pyruvic acid from carbon dioxide. Any intermediate compound should act as a substitute for photosynthesis provided that the compound can pass through the cell membrane. Most phosphorylated compounds are unable to do so. Alternatively, if an organism is able to phosphorylate a substrate such as pyruvic acid or glucose, or is able to phosphorylate a product of the metabolism of such a substrate, then that substrate should be able to substitute for photosynthesis. Among the energy sources which have been found suitable for the growth of various plant-like flagellates are lower fatty acids, some alcohols, lactic acid and pyruvic acid. In some flagellates acetic acid is the only compound which can be used as a source of energy, and in all known cases where a variety of organic compounds can be utilised, acetic acid is one of these compounds. Since obligate phototrophs can not utilise acetic acid, it seems clear that acetic acid itself is not an intermediate in the starch synthesis. In Scheme I it is postulated that phosphopyruvic acid is synthesised from a phosphorylated two-carbon compound, which could be acetyl phosphate or phosphoenol acetic acid, and a monocarbon compound such as formic acid. In obligate phototrophs, acetyl phosphate can not be synthesised from acetic acid otherwise acetic acid would be utilisable as an energy source;

KEY TO SCHEME I

Enzymes responsible for reactions 1 - 10 are:-

1) Enolase

2) Phosphoglyceromutase

3) Phosphoglyceric phosphokinase

4) Triosephosphate dehydrogenase

5) Phosphotriose isomerase

6) Aldolase

7) Phosphohexokinase and hexose diphosphatase

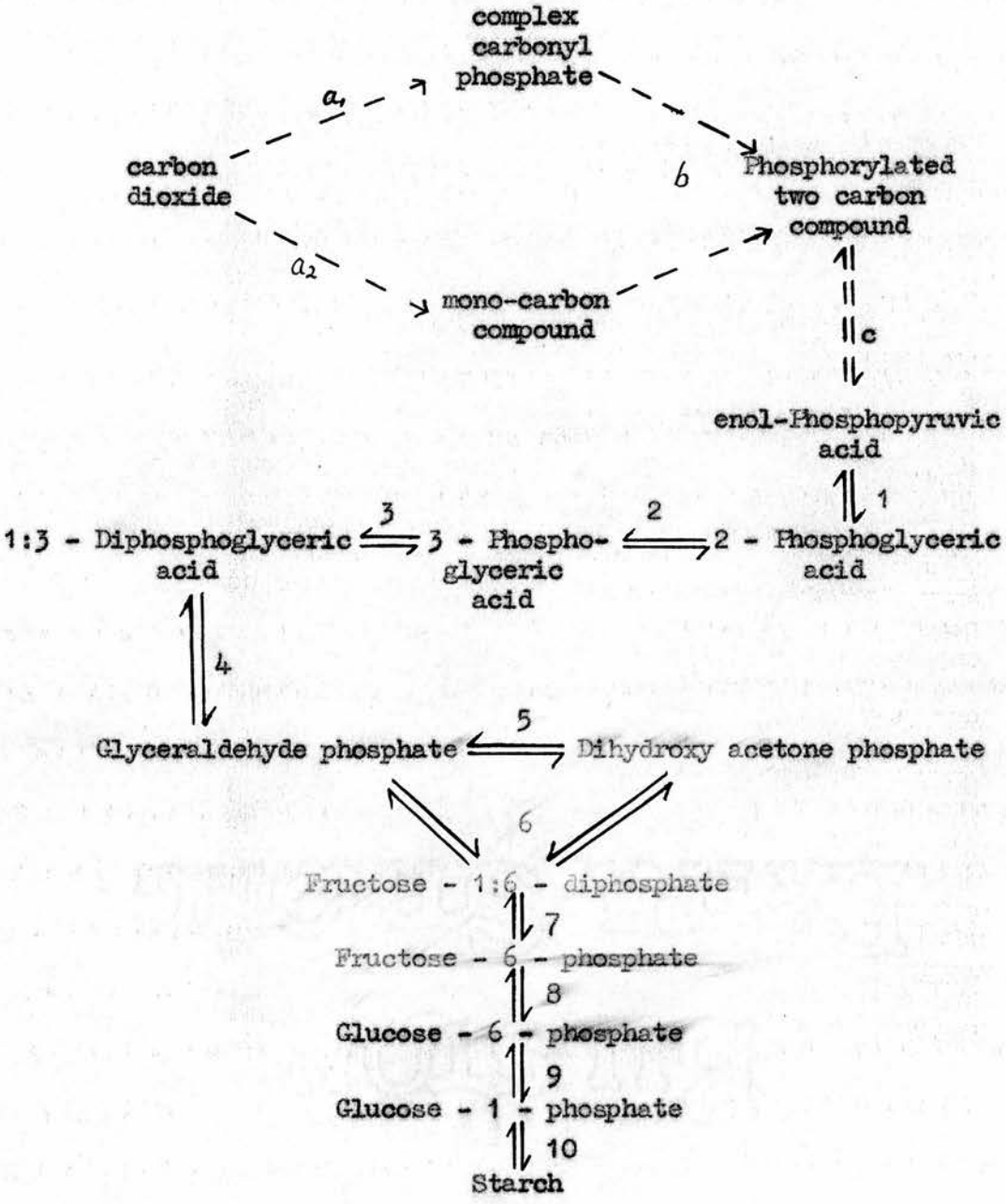
8) Phosphohexoisomerase

9) Phosphoglucomutase

10) Phosphorylase (with branching and debranching enzymes).

SCHEME I

SYNTHESIS OF STARCH FROM CARBON DIOXIDE (after A. LWOFF (3))



phosphorylation must therefore take place at a very early stage. Ruben (12) has suggested that one of the first steps in photosynthesis is the formation of a complex carbonyl phosphate $R{\cdot}COOH_2PO_3$. This would act as an acceptor of a monocarbon carbon compound in the synthesis of phosphorylated acetic acid.

The postulated mechanism of glycogenesis (Scheme I) is consistent with the above observations. Obligate phototrophy is due to an inability to phosphorylate any intermediate between the complex carbonyl phosphate and starch and is, therefore, due to a lack of phosphokinases. The ability of certain phototrophs to survive in the absence of light when placed in an acetate-containing medium may be regarded as the first stage in the transition from plant-type to animal-type modes of nutrition. This ability is seen to be due to the synthesis by the organism of the necessary phosphokinases. In later stages of this transition, enzyme systems are present which can metabolise increasingly complex organic compounds, and in the final stage the organism is capable of ingesting particulate food of extreme complexity.

(b) Intracellular Polysaccharides other than Starch

Several free-living and intestinal protozoa have been reported (2) (13) (14) to synthesise, and to store intracellularly as food reserve materials, glucose containing polysaccharides which stain brown with iodine and which were therefore described as glycogens. The first such glycogen to be investigated chemically was that synthesised by the free-living ciliate, Tetrahymena pyriformis (15). Pure cultures of this protozoan, grown in a peptone medium of low carbohydrate content, gave cells having a polysaccharide content of 16 - 20% (on a dry weight basis). The purified polysaccharide showed all the properties of a typical animal glycogen. Evidence that polysaccharide synthesis could proceed according to Scheme I (reactions 6-10)

was obtained by the detection, (16) in cell free extracts of these protozoa, of aldolase, phosphohexokinase, oxoisomerase, phosphoglucomutase and phosphorylase activities.

Polysaccharides isolated from Trichomonas foetus (17), Trichomonas gallinae (17), Cycloposthium (18) (19) and by the holotrichously ciliated Isotricha and Dasytricha of sheep's rumen (20) have also been examined; those isolated from the parasitic flagellates T. foetus and T. gallinae were generally similar to typical animal glycogens though they showed some differences in branching properties. The polysaccharide isolated from the holotrich ciliates of sheep's rumen resembled that of the ciliate Cycloposthium and was closely similar to a typical plant amylopectin.

The differences in the degree of branching in amylopectin-type and glycogen-type polysaccharides presumably reflect differences in the enzyme systems catalysing the synthesis of these polysaccharides. In T. gallinae the ratio of the activities branching enzyme:phosphorylase must be greater than in T. foetus or T. pyriformis and much greater than in Cycloposthium and the holotrich ciliates of sheep's rumen.

IV Scope of the Present Work.

The present work has been mainly concerned with the structure of protozoal polysaccharides and the metabolism of oligosaccharides by extracts of Tetrahymena pyriformis.

Since the majority of protozoal polysaccharides hitherto examined have belonged to the amylopectin-glycogen class, preliminary studies have been directed towards semi-micro methods of end-group assay by periodate oxidation and to quantitative measurements of the absorption spectrum of polysaccharide - iodine complexes. For these experiments polysaccharides from T. pyriformis,

T. foetus and T. gallinae have been used, together with authentic specimens of animal glycogen and plant amylopectin.

The main part of the work has been concerned with the examination of the polysaccharides synthesised by the flagellates Chilomonas paramecium and Ochromonas malhamensis.

In view of current interest in the metabolism of oligosaccharides and in transglycosylation reactions, the metabolism of maltose and of cellobiose by cell-free extracts of Tetrahymena pyriformis has also been investigated.

SECTION II

GENERAL METHODS

The following general methods were employed in the present investigations. Numbered methods are referred to as such in the course of the text.

Paper Chromatographic Methods

The following solvents and spray reagents were used for the separation and preliminary identification of carbohydrates by paper partition chromatography. Separation was effected by the method of descending flow on Whatman No. 1 paper at room temperature. The R_G values (i.e. movement relative to D - glucose) of the various carbohydrates are recorded, where appropriate, in the text.

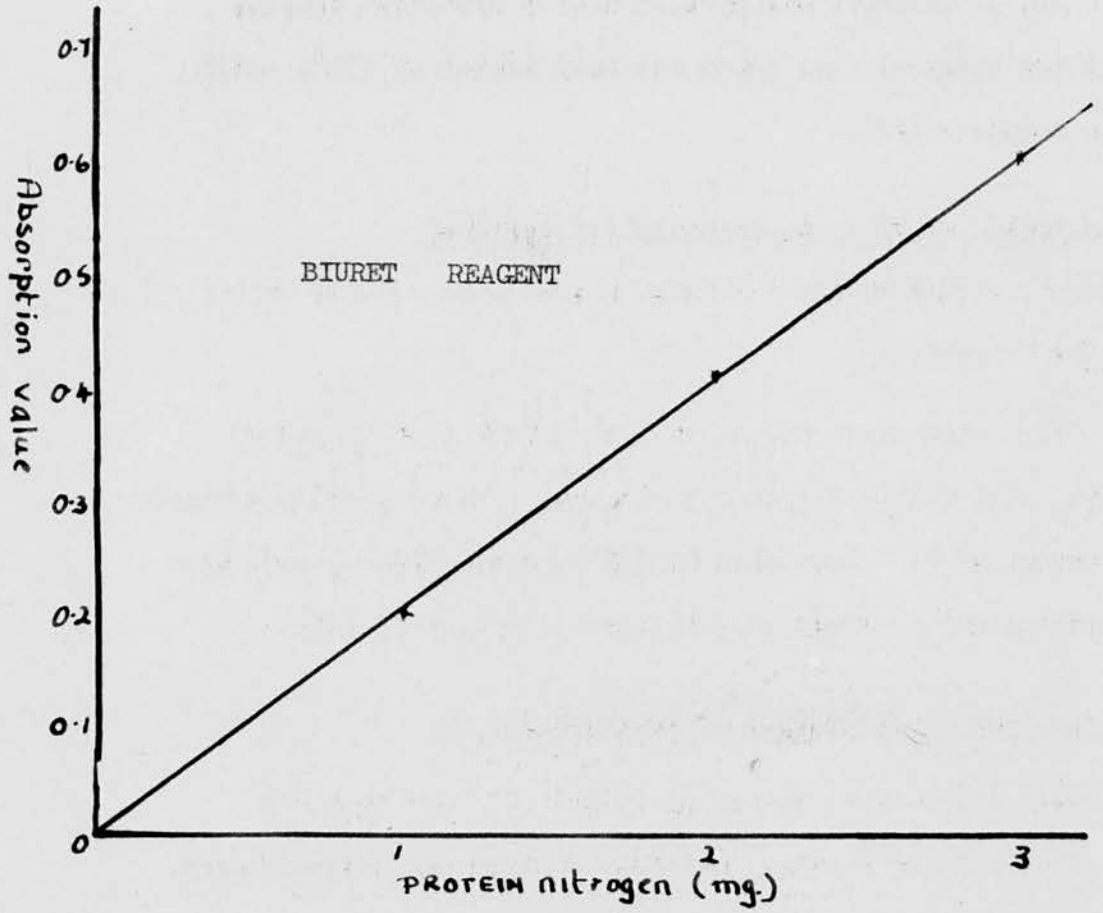
Separation of oligosaccharides

Solvent (1): ethyl acetate - pyridine - water (10:4:3)

Solvent (2): butanol - pyridine - water (6:4:3)

Spray (1). The dried paper was passed through a solution prepared by adding saturated aqueous silver nitrate solution (1 ml.) to acetone (200 mls.) and adding water dropwise with shaking until the precipitated silver nitrate re-dissolved. After drying, the paper was sprayed with a 0·5 N - solution of sodium hydroxide in aqueous ethanol. Excess reagent was removed by washing in 6 N - ammonium hydroxide solution and then water (21).

CALIBRATION GRAPHS OF SOMOGYI AND BIURET REAGENTS



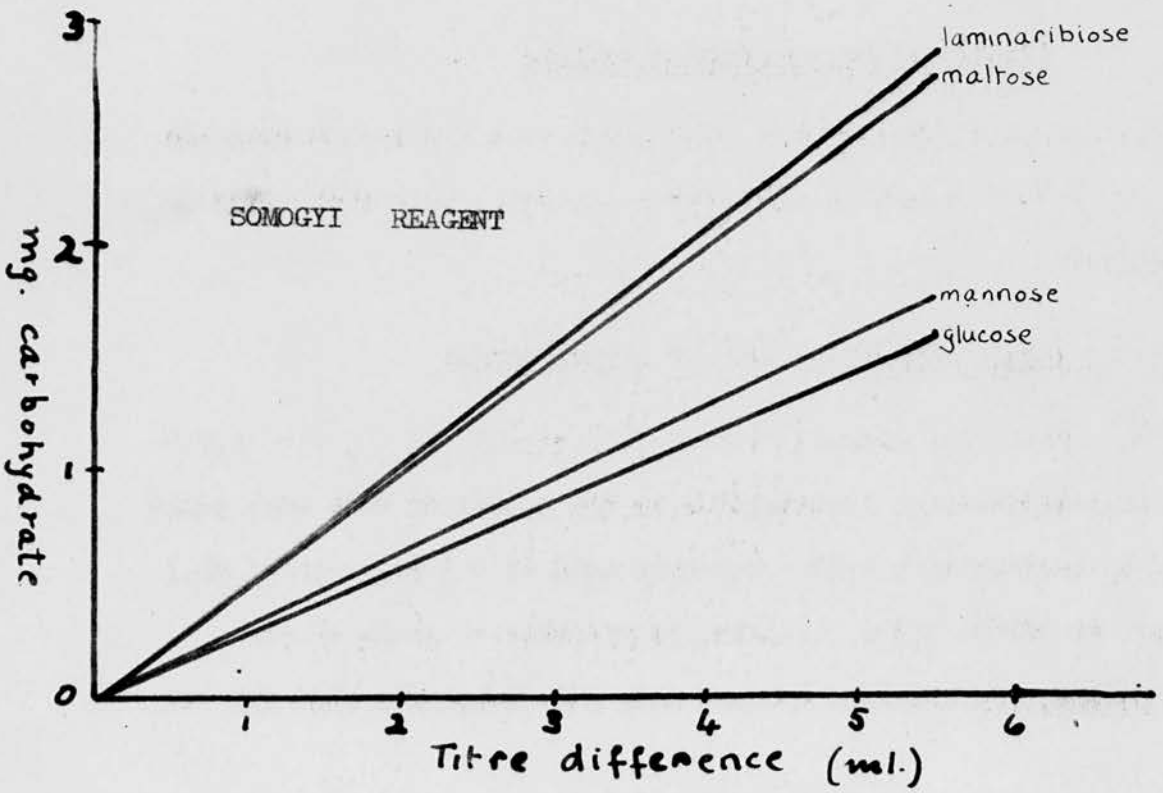


Fig 2.

Spray (2). The dried paper was sprayed with a saturated aqueous solution of aniline oxalate; the paper was then heated at 130°C until development was complete (22).

Separation of glucose, mannitol and sorbitol

Solvent (3): ethyl methyl ketone - glacial acetic acid - water (9:1:1), saturated with boric acid (23)

Spray (3). The dried paper was sprayed with 0·6% aqueous sodium metaperiodate solution followed (after 5 minutes) with a solution prepared by mixing 10 volumes of 0·1 M benzidine in 50% aqueous ethyl alcohol with 2 volumes of acetone and 1 volume of 0·2N hydrochloric acid (24).

Quantitative Estimation of Reducing Sugars

The Somogyi cuprimetric titration method (25) was used for quantitative estimations of glucose, mannose, maltose and laminaribiose. Calibration graphs for these sugars are shown in Fig. 2.

Drying of Polysaccharide Samples

Polysaccharide samples were dried prior to quantitative analysis by heating for several hours at 60°C over phosphorous pentoxide under reduced pressure.

Complete Acid Hydrolysis of Carbohydrates

It was found that complete acid hydrolysis of all the α - linked oligo- and poly-saccharides investigated in the course of this work could be effected by heating with 1·5N - sulphric acid (1 - 5 mg. sugar / ml.) for two hours at 100°C. The β-linked polysaccharide isolated from Ochromonas malhomensis required heating with 2N - sulphuric acid for two

hours at 100°C for complete hydrolysis. Acid degradation of glucose, determined by means of the Somogyi reagent (25) was found to be negligible during this treatment.

Method 1. Estimation of Ash Content.

Weighed samples of polysaccharide (20 - 50 mg.) were ignited to constant weight in a platinum crucible and the weight of the resultant ash determined.

Method 2. Estimation of Protein Nitrogen.

Protein nitrogen was determined by the method of Robinson and Hogden (26); lyzozyme (which had 14·1% N by the Kjeldahl method (27)) being used as a standard.

(i) Reagents:-

10% trichloroacetic acid solution

3% sodium hydroxide solution

20% copper sulphate (pentahydrate) solution.

(ii) Calibration

To each of 1·0 and 2·0 and 3·0 mls. of standard lysozyme solution (7·26 mg./ml.) in graduated centrifuge tubes was added sodium hydroxide reagent to give a total volume of 9 ml. Copper sulphate solution (0·25 ml.) was added and the volume made to 10 mls. with the alkali. After shaking (1 minute) the mixtures were allowed to stand (15 minutes). The colours of the supernatant solutions after centrifugation were compared on a Spekker Photoelectric Absorptiometer in 1 cm. cells at 550 mμ against a water blank. The following results, corrected for a reagent blank, were obtained and are graphically represented in Fig. 2.

Protein nitrogen (mg.)	1·01	2·02	3·03
Absorption Value	0·196	0·409	0·601

These results were very similar to those obtained by other workers in these laboratories.

Periodate Oxidations

Method 3. Determination of Periodate Uptake.

The uptake of periodate during the oxidation of carbohydrates with sodium metaperiodate was determined by the method of Fleury and Lange (28) in which samples of the reaction mixture are mixed with standard arsenite solution in the presence of potassium iodide and sodium bicarbonate; excess arsenite is determined by back titration with iodine.

Method 4. Determination of Formic Acid Release.

The production of formic acid during sodium- and potassium- periodate oxidation of carbohydrates was determined after neutralisation of excess periodate with ethylene glycol (0·5 - 2·0 ml., depending on the amount of residual periodate) by titration with approximately 0·01N standard carbonate free sodium hydroxide using (a) methyl red indicator or (b) a glass electrode and a Pye Universal pH meter to an end-point at pH 5·8.

Determination of Formaldehyde

The colorimetric determination of formaldehyde by means of chromotropic acid (29) was examined and a calibration graph prepared. However, when this method was employed to estimate the production of formaldehyde on periodate oxidation of maltose a strong green fluorescence developed and the observed intensities were in excess of theoretical. It was later found (30) that colour intensities produced on periodate oxidations

of polysaccharides were also far in excess of the expected values. It was considered that the concentrated sulphuric acid, which comprised five sixth of the reagent caused the production of interfering furfural.

Method 5. The micro-method of formaldehyde estimation in the presence of periodate, developed by Hough, Powell and Woods (31) was then employed.

Reagents: (a) 2 volumes of aqueous 2% solution of 'A.R.' potassium ferricyanide mixed with 5 volumes of concentrated hydrochloric acid (S.G. 1·16).

(b) 1% solution of purified phenylhydrazine hydrochloride in 0·2 - sodium acetate - acetic acid buffer, pH 3·5.

(c) 4 volumes of saturated aqueous barium chloride solution were mixed with 1 volume of saturated aqueous sodium bicarbonate solution; the resulting precipitate was removed by centrifugation.

Reagents (a) and (c) were prepared as required.

Calibration of reagents.

The reagents were calibrated under conditions identical to those encountered in the periodate oxidation studies.

(a) Unbuffered Conditions. To each of 0, 1, 2, 3, 4 and 5 ml. standard formaldehyde solution 191·4 μ.g / ml; concentration determined by means of standard iodine and sodium thiosulphate solutions (32) , was added sodium metaperiodate solution (2 ml; 0·3 M). The mixture was diluted to 25 mls. with water and the solutions stored in the dark at room temperature for twenty-four hours. Aliquots (2 ml.) were withdrawn and mixed with reagent (c) (2 ml.), and, after standing, the mixtures were clarified by centrifugation. Aliquots of the supernatant solutions (2 ml.) were mixed with reagent (b) (2 ml.) in 50 ml. graduated flasks and set aside in the

CALIBRATION GRAPH OF FORMALDEHYDE REAGENT.

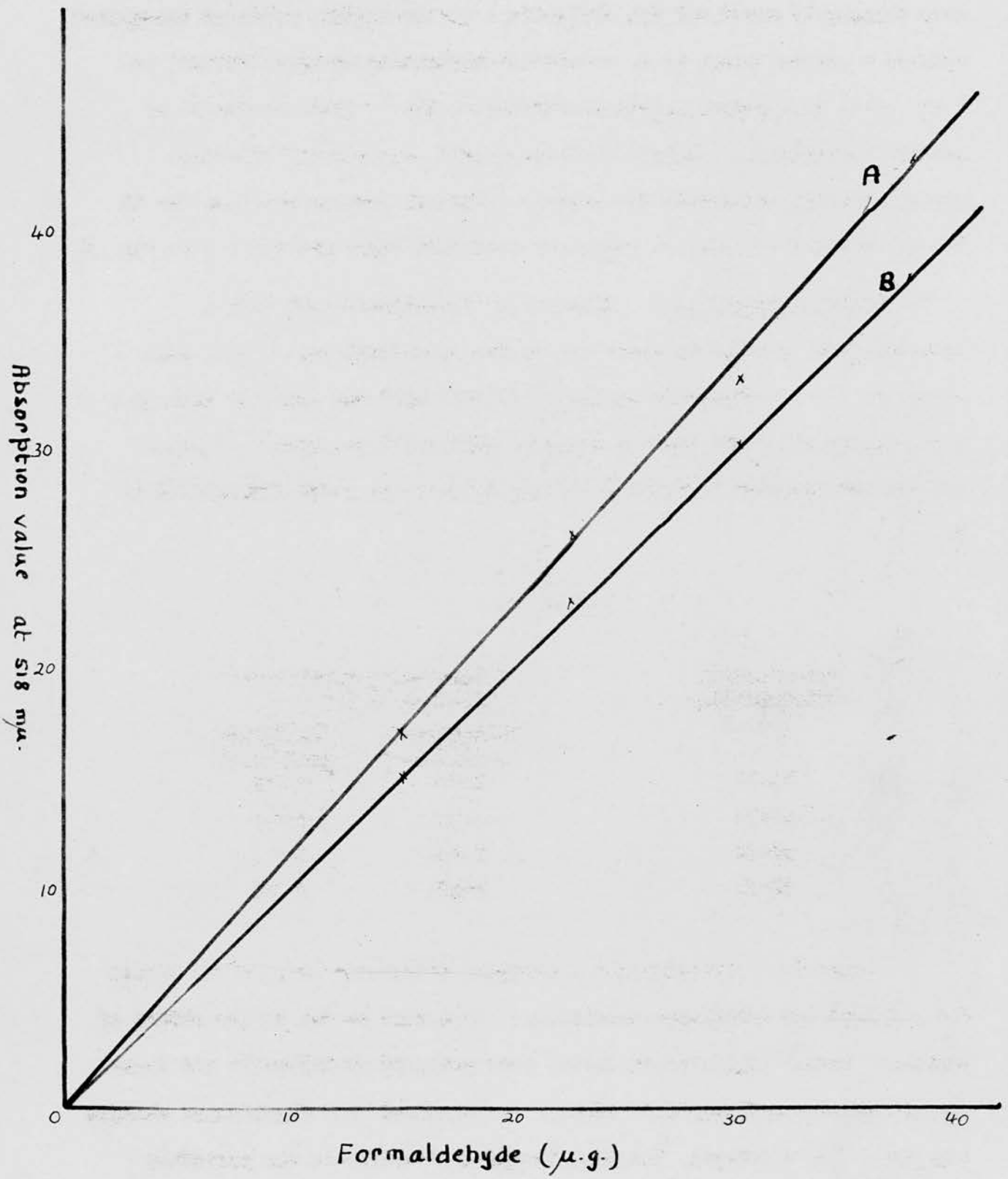


Fig. 3

dark for 30 minutes. Reagent (a) (7 ml.) was then added and, after 3 minutes, the mixtures were diluted to 50 ml. with water. The solutions were thoroughly mixed and the absorptions of the colours produced determined against a reagent blank (i.e. a solution containing no formaldehyde) in 1 cm. cells in a Unicam Spectrophotometer at 518 mμ (the wavelength of maximum absorption). Slight variations were apparent for different phenylhydrazine hydrochloride reagents: typical results are recorded in Table 3 and the calibration graph produced from them, represented in Fig. 3.

(b) Buffered Conditions. Mixtures of formaldehyde and sodium metaperiodate, similar to those set up for unbuffered conditions, were prepared; 0·1M - phosphate buffer pH 8 (12·5 ml.) was added to each in a total volume of 25 ml. and the reagents calibrated as above. Typical results are recorded in Table 3 and the calibration graph represented in Fig. 3.

Table 3

Formaldehyde Concentration (μg.)	Unicam Spectrophotometer Reading at 518 mμ.	
	Unbuffered conditions	Buffered conditions
15·30	0·170	0·150
22·95	0·260	0·230
30·60	0·330	0·300
38·25	0·431	0·375

There is a significant and constant difference between the values for buffered and unbuffered conditions; this must be due to the effect of phosphate ions. Calibration curves were prepared at intervals and fresh phenylhydrazine hydrochloride reagent was prepared when significant changes occurred. As a control, the formaldehyde production on the periodate

oxidation of glucose under the unbuffered conditions was determined; this was equivalent to 1·01 moles formaldehyde per mole of glucose which is in good agreement with the theoretical value.

SECTION III

END-GROUP ASSAY OF GLYCOGEN-TYPE POLYSACCHARIDES BY OXIDATION WITH SODIUM METAPERIODATE

I. INTRODUCTION

Periodic acid oxidation was first applied to the study of carbohydrates by Malaprade (33) who found that in the oxidation of sugar alcohols of general structure $CH_2OH \cdot (CHOH)_n \cdot CH_2OH$, $(n + 1)$ moles of periodate were consumed with the production of two moles of formaldehyde and n moles of formic acid.

Periodate oxidation techniques have found wide application in structural studies in carbohydrate chemistry (34) (35) (36). Jackson and Hudson (37) showed that periodic acid would react with carbohydrate molecules which were stabilised in the ring formation: α- and β-methyl glucosides (and other methyl glycosides) were oxidised yielding one mole of a dialdehyde together with one mole of formic acid. This work suggested that periodate oxidation could be used as a means of assay of 1:4-linked glucosans. Each glucopyranose radical at an intermediate position in the polymeric chain has an α-glycol group at carbon atoms 2 and 3, and will consume one mole of periodate with the formation of a dialdehyde. Non-reducing terminal radicals will have free hydroxyl groups on carbon atoms 2, 3 and 4, and will react with two moles of periodate, one mole of formic acid being liberated (cf. Fig. 4). Measurement of the amount of formic acid released on periodate

oxidation of branched glucosans such as glycogen and amylopectin should therefore provide an accurate means of end-group assay. Owing to the size, and the high degree of branching, of such molecules, the reaction of the periodate ion with the single reducing group (which can give two moles of formic acid) may be neglected.

Early workers found that during oxidation of 1:4-glucosans the periodate uptake and the release of formic acid were often greater than expected. This effect was termed overoxidation (38). Conditions which avoided such overoxidation and which were therefore suitable for quantitative studies were first described by Hirst and his co-workers. In 'model' experiments using methyl glycosides of mono- and di- saccharides these workers found that quantitative oxidation could be effected in 150 hours by the use of sparingly soluble potassium periodate at room temperature (39). Under these conditions end-group assays of various polysaccharides gave results which agreed fairly well with those obtained from methylation studies (40) (41).

Bell and Manners in 1952 reported that, on oxidation of glycogens with potassium periodate, production of formic acid did not cease within 150 hours, and that chain length values based on the 150 hour figure were incorrect (42). They suggested that a large branched polysaccharide molecule was oxidised less rapidly than were small molecules such as β-methyl maltoside. The reaction was therefore allowed to proceed beyond 150 hours and it was found that liberation of formic acid came to completion after 250 - 300 hours. The maximum yield of formic acid was used in calculation of the average chain length. These observations are supported by those of Anderson, Greenwood and Hirst who examined the potassium periodate oxidation of starches (43).

Other methods of periodate oxidation of glucosans have been suggested. Potter and Hassid in 1948 criticised the potassium periodate method on the grounds that it was too slow (45). These authors approached the problem of end-group assay by studying first the periodate oxidation of maltose. Their aim was to find conditions under which maltose would yield three moles of formic acid and then to apply these conditions to the end-group assay of polysaccharides. Potter and Hassid found that, by carrying out the oxidation of maltose with sodium periodate in sodium chloride solution at 2°C, formic acid production was rapid until, after 25 hours, the theoretical value of three moles was reached. However, the reaction did not stop completely; the production of formic acid continued slowly, due mainly to overoxidation of the reducing end-group. Nevertheless in experiments in which amylose and amylopectin were oxidised, the amount of formic acid produced in 25 hours was used in calculation of the proportion of end-groups. The method is therefore based on the unproved assumption that oxidation of the disaccharide, maltose, proceeds at the same rate as that of a polysaccharide of molecular weight ca. 10^7 Potter and Hassid reported chain lengths of 22 - 27 for various amylopectins although these values were not confirmed by methylation assay of the same samples. Later workers have reported that the C.L. values of several amylopectins and glycogens obtained by periodate assay under these conditions were greater than those obtained by other methods of assay on the same samples (46) (47) (48).

Meyer and Rathgeb (49) studied the periodate oxidation of both simple sugars and glucosans. They found that sodium periodate was a satisfactory oxidant provided that the temperature of the reaction was kept at 0°C, and the pH between 4·2 and 5·8. Under these conditions the authors

found that the oxidation of branched glucopolysaccharides such as amylopectin was complete in 150 - 200 hours.

Results of end-group assays on a number of glycogens were published in 1951 by Abdel-Akher and Smith (50) who claimed that, with a low concentration of periodate and a low temperature, overoxidation was avoided.

The effect of light on periodate oxidation has been studied by Head and his co-workers (51) (52) (53) who showed that although normal Malapradian oxidations are not noticeably accelerated by exposure to light, the rate of overoxidation, including the further oxidation of formic acid, is greatly increased. The possibility of oxidative destruction of formic acid was also investigated by Sarkar (54) who noted that, in the presence of periodate, oxidation of formic acid occurred when it was present in fairly high concentrations. At concentrations of 0·002 N. or less, no loss of formic acid could be demonstrated.

It is therefore apparent that overoxidation can be controlled by carrying out the reaction in the dark and by keeping both the concentration of periodate in the reaction mixture, and the concentration of formic acid formed during the reaction, low. Under these conditions, overoxidation is negligible. Furthermore, loss of formic acid does not occur.

In the present work the oxidation of maltose under the conditions described by Potter and Hassid (45) was examined. In the course of this investigation it was found that, presumably due to some interaction between the sodium chloride and the sodium periodate, the reagent blank became appreciably acid and a periodate complex precipitated from solution. Since much of the periodate in the maltose oxidation mixture is consumed in the oxidation of maltose, there is uncertainty as to the amount of acid produced

in such mixtures by the interaction of periodate and sodium chloride; the accurate estimation of the formic acid liberated from the maltose is, therefore, not possible. This source of error must also be present in the oxidation, in the presence of sodium chloride, of glycogen. The procedure of Potter and Hassid has, therefore, been modified, sodium chloride being omitted from the reaction mixture. End-group assay of several glycogens, including some of protozoal origin, has been carried out using this modified procedure.

II DISCUSSION

Oxidation of Maltose

Samples of maltose were oxidised under the conditions described by Potter and Hassid (45) namely, oxidation at 2°C in a solution containing 1·1% maltose, 4% sodium metaperiodate and 3% sodium chloride., formic acid production and periodate uptake being determined at intervals. Reagent blank solutions (mixtures of sodium chloride and sodium metaperiodate) were also prepared; these became acid, the pH falling to ca. 3·0. This acidity was accompanied by the precipitation of periodate from solution. Such precipitation also occurred in the maltose oxidation mixtures although to a lesser extent. The development of acidity due to the interaction of the sodium chloride and the sodium metaperiodate may also proceed in the maltose oxidation mixture. Determination of periodate uptake and the accurate estimation of the formic acid arising from the oxidation of the maltose is, therefore, not possible. Approximate results may be obtained by the initial (2 - 4 hr.) values of the reagent blank. Solutions containing 3% sodium metaperiodate and 3% sodium chloride or 4·4% sodium nitrate were prepared and placed in the dark at 2°C. Precipitation took place in each solution and the pH of each fell to 3·0. Aliquots were removed at intervals into ethylene glycol and titrated with sodium hydroxide. The titres increased with time. A blank solution containing only sodium metaperiodate (3%) did not develop acidity; no precipitation occurred from this solution. It seems probable that the precipitation which occurs in the presence of sodium chloride or sodium nitrate is due to a common ion

effect at the limit of the solubility. A further difficulty which prevented the accurate estimation of formic acid release was the tendency of the neutralised solutions to become acid on standing for a few minutes; this is discussed later.

In several experiments the formic acid production from maltose at 25 hours varied between 2·2 and 2·5 moles, the corresponding periodate uptake being 4·2 - 4·5 moles. After 72 hours the formic acid release was 2·9 - 3·1 moles, the periodate uptake being 4·5 - 5·1 moles.

The observation of Potter and Hassid (45) that, under these conditions, the periodate oxidation of maltose is complete after 25 hours and results in the liberation of 3 moles of formic acid, could not, therefore, be confirmed. From the present results it appears that oxidation is complete only after 72 - 144 hours. The periodate uptake required for complete Malapradian oxidation is 5 moles. These results are similar to those obtained in the oxidation of lactose (55) and cellobiose (56) suggesting that, like these latter sugars, the oxidation of maltose proceeds by hydrolysis of the formyl ester (Fig. 4).

In view of the uncertainty caused by the presence of sodium chloride, the oxidation of maltose was repeated in aqueous solution. In several experiments the formic acid release, after 25 hours was 2·2 - 2·4 moles, the periodate uptake being 4·2 - 4·5 moles. After 72 hours these values were 2·5 - 2·8 moles and 4·4 - 4·8 moles respectively. In one such experiment, the formaldehyde release was also determined and after 25 hours, was equivalent to 0·29 moles. This increased to 0·95 moles after 144 hours.

In the above oxidations it was observed that the neutralised (sodium hydroxide) solutions tended to become acid on standing. In addition, the

PERIODATE OXIDATION OF MALTOSE

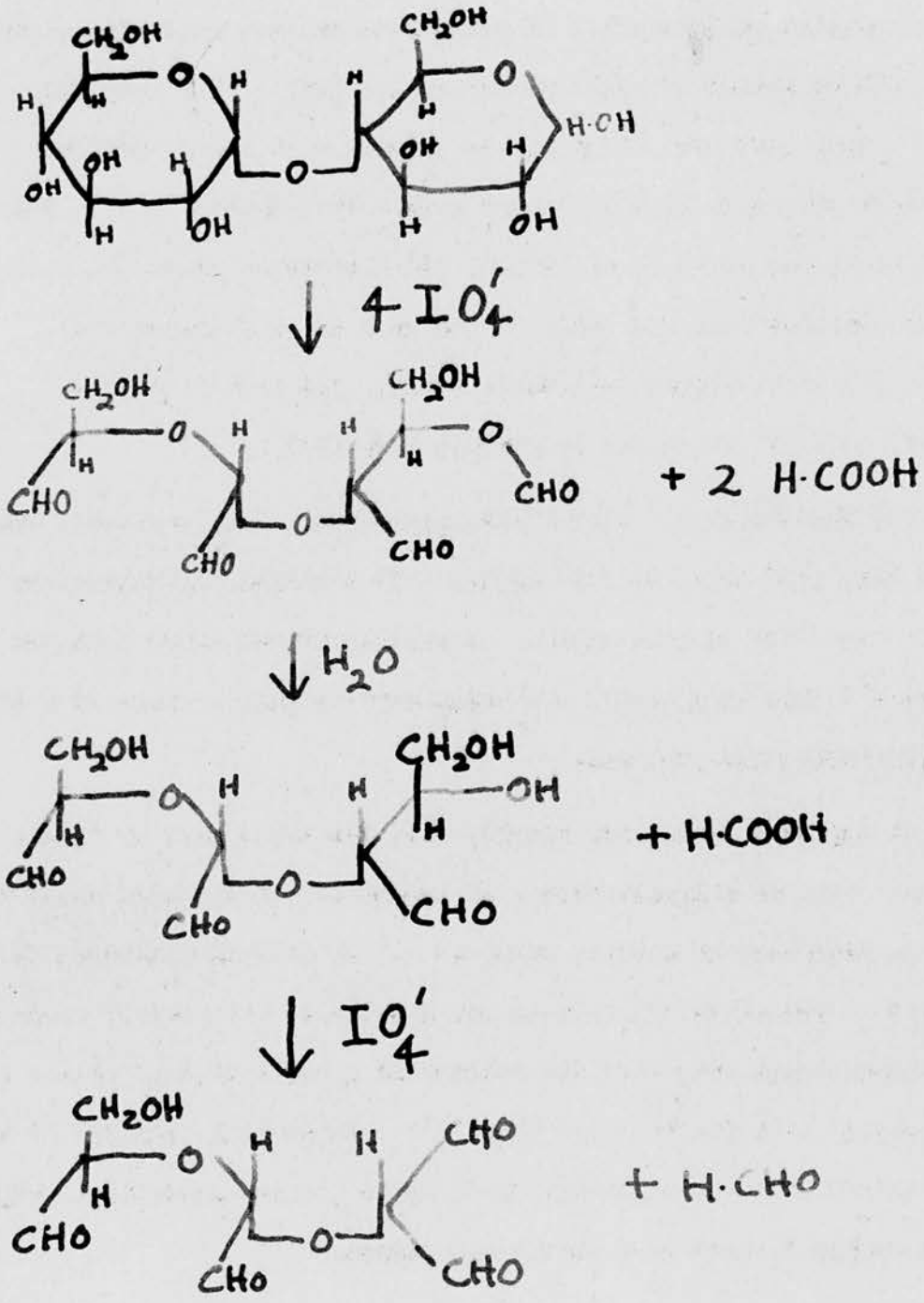


Fig. 4.

volume of sodium hydroxide required for neutralisation was influenced by (a) the time allowed for the destruction, by ethylene glycol, of the periodate in the sample, and (b) the speed of titration. In quantitative experiments, samples removed after 48 hours from maltose oxidation mixtures were mixed with an excess of ethylene glycol and left in the dark for 30 minutes. They were then titrated, as before, with carbonate-free 0·01 - N - sodium hydroxide in a stream of carbon dioxide-free air. The flasks containing the neutralised (methyl red indicator) solutions were then tightly stoppered and set aside in the dark at room temperature. On standing the solutions became noticeably acidic, and 0·01 N - sodium hydroxide was added at intervals to restore neutrality.

In one experiment the formic acid production, thus measured, rose in 24 hours from 2·56 moles to 2·98 moles. In a second experiment the increase was from 2·39 to 2·71 moles. A sample removed after 72 hours oxidation at 2°C from this latter oxidation mixture gave a value of 2·61 moles for the formic acid release.

It is apparent from these results that the third mole of formic acid does not arise as a direct result of oxidation and it seems clear that the periodate oxidation of maltose involves (a) an initial oxidation in which 4 moles of periodate are reduced and 2 moles of formic acid produced, and (b) a slower stage involving the release of a third mole of formic acid by the hydrolysis of a formyl ester (Fig. 4). Evidence in support of this was also obtained by the observation that, under similar conditions, ethyl formate and propyl formate were slowly hydrolysed.

Oxidation of Glycogen

Because of the acidity arising from the interaction of the sodium

chloride and the sodium metaperiodate, accurate determination of the formic acid release from glycogen, oxidised under the conditions of Potter and Hassid, was not possible. Furthermore, because of the relatively low formic acid release, this error will be much greater than in the oxidation of maltose. For this reason the method of Potter and Hassid has been modified. Glycogen samples have been oxidised at 2°C in aqueous solution with a 20% excess of sodium metaperiodate. Aliquot samples were analysed at intervals until constant values were obtained for formic acid production. C.L. values are obtained from the formic acid release by substitution in the equation

$$F = \frac{46 \times 100}{180 + (n - 1)162}$$

F = formic acid release (%)

n = C.L.

The C.L. values of several glycogens were also determined by oxidation with potassium periodate (p. 40) at room temperature.

Collected results are given in Table 4. It is of interest to note that the degree of branching in the second preparation of T. gallinae glycogen differs slightly from that reported by Manners and Ryley (17) for the original sample, although the value of this second sample and that of the second sample of T. pyriformis glycogen is similar to most animal glycogens.

It was found that oxidation by sodium periodate was complete after 8 - 11 days (p.41). C.L. values thus obtained for 8 samples of glycogen were in good agreement.with values obtained from potassium periodate oxidation.

However, two samples (rabbit liver X and cat liver VI) gave C.L. values which were far in excess of the theoretical. These samples were

TABLE 4

End-Group Assay of Glycogen by Periodate Oxidation

Glycogen sample	Oxidation method		Previously reported value. (42) (57) (58)
	Potassium periodate	Sodium periodate	
Rabbit liver I	-	13	13
Rabbit liver V	12	13	12
Rabbit liver X	12	12	12
Rabbit liver XX	13	12	-
Rabbit liver XXI	-	13	-
Tetrahymena pyriformis II	14	13	-
Trichomonas gallinae II	13	13	-
Ascaris lumbricoides	-	12	12
Human muscle	-	11	12
Cat liver VI	-	12	12
Amylopectin sample			
Rice	-	19	-
Waxy maize starch II	-	21	21
Waxy maize starch IV	-	22	23

again oxidised, the conditions being varied slightly so that, although the ratio of periodate to glycogen remained the same, the concentration of the reactants was increased. The formic acid production and the periodate uptake were measured at intervals. It was found (p.43) under these conditions that, with rabbit liver X, the periodate uptake after 14 - 21 days was 1·07 - 1·11 moles (theoretical, 1·08 moles). The C.L. value obtained from the formic acid production at this time was 12·6 - 11·9 (previously reported value, 12). After 28 days oxidation, the C.L. value was 10·8; the periodate uptake, 1·20 moles, showed that overoxidation had occurred. With cat liver VI glycogen, oxidation was incomplete at 28 days. The periodate uptake at this time was 1·06 moles, the C.L. value being 13·6 (previously reported value, 12).

These two samples were again oxidised. The periodate concentration was increased (50% excess) and the oxidation was carried out in the dark at room temperature. The formic acid release from rabbit liver X glycogen was constant at 8 - 10 days. The C.L. value thus obtained was 11·9, the periodate uptake being 1·07 - 1·08 moles. At 15 days oxidation the formic acid production had increased due to overoxidation.

With cat liver VI glycogen, the formic acid production reached constancy at 13 - 17 days. At this time the C.L. value and periodate uptake were 11·9 units and 1·09 moles respectively.

From the collected results it is seen that oxidation at 2°C with a 20% excess of periodate is generally complete after 8 - 11 days. C.L. values thus obtained are in good agreement with the results of potassium periodate oxidation assay of the same samples. Two of the glycogen samples examined did, however, require a much longer time for complete oxidation under these conditions. Differences in the rate of oxidation of various glycogen

samples have previously been reported (42) (59). Such differences were considered to be caused in part, by variation in the temperature at which the oxidation was carried out (60). In the present work it has been found that, under identical conditions, certain glycogens are oxidised more slowly than others. The reasons for the differing susceptibilities to oxidation are not known.

C.L. values determined by sodium metaperiodate oxidation are, however, unambiguous since, after the completion of the primary oxidation, the production of formic acid stops or proceeds at a very much lower rate. Measurement of periodate uptake is a convenient method of following the extent of oxidation and measurement of the formic acid release when the periodate uptake is ca. 1·08 moles gives theoretically correct values of C.L.

The two samples of glycogen, rabbit liver X and cat liver VI, which were incompletely oxidised under the conditions used for the other samples, gave, when the oxidation was allowed to proceed until the periodate uptake reached ca. 1·08 moles, values of C.L. which were in very good agreement with the previously reported values.

It is seen from the collected results (Table 4) that oxidation, under the present conditions, with sodium metaperiodate provides a satisfactory method of end-group assay. This method has certain advantages over the potassium periodate method (39) (42). Assay by potassium periodate oxidation normally requires ca. 200 mg. of polysaccharide and, since the oxidation mixture is heterogeneous, there is some uncertainty in sampling. Furthermore, for the determination of periodate consumption it is necessary to set up small individual oxidation mixtures and to analyse each complete mixture. With sodium periodate oxidation on the other hand end-group assay

may conveniently be effected on 50 - 100 mg. polysaccharide; the oxidation mixture is homogeneous and the course of the reaction can be followed by determination of the periodate consumption; analysis of the formic acid release when the periodate consumption is constant, or is equivalent to ca. 1·08 moles of periodate per an-hydroglucose unit, gives C.L. values which are in good agreement with the values obtained by potassium periodate oxidation or by methylation. It is normally difficult to obtain large quantities of polysaccharide from microbiological sources and the sodium periodate oxidation method of assay is therefore of especial value in the examination of such polysaccharides.

Conclusions

1. The periodate oxidation of maltose involves (a) an initial oxidation in which 4 moles of periodate are consumed and 2 moles of formic acid produced, (b) a slower stage involving the release of a third mole of formic acid by hydrolysis of a formyl ester, and (c) a further oxidation in which 1 mole of periodate is consumed and 1 mole of formaldehyde produced.

2. Under the conditions described by Potter and Hassid (45), oxidation of maltose is incomplete after 25 hours and the corresponding reagent blank develops acidity. This prevents the accurate estimation of formic acid produced by the oxidation.

3. The oxidation of glycogen-type polysaccharides at 2°C with a 20% excess of periodate is generally complete after 8 - 11 days. The course of the reaction may be conveniently followed by measurement of the periodate consumption. C.L. values calculated from the formic acid release at complete oxidation are in good agreement with values obtained by potassium

periodate oxidation assay.

4. Different samples of glycogen are oxidised, under identical conditions, at different rates.

III EXPERIMENTAL

Sodium Metaperiodate Oxidation of Maltose

(1) Oxidation in presence of sodium chloride (45).

The concentration of an approximately 2% solution of recrystallised maltose in 3% aqueous sodium chloride was determined, after allowing for the mutarotation of the maltose, by polarimetry (2 dm. tube). Aliquots of this solution (10 ml.) were cooled to 2°C and mixed with 0·37 M - sodium metaperiodate solution (10 ml.). A reagent blank was also prepared. The two solutions were kept, in the dark, at 2°C. Aliquots (1 ml.) were removed at intervals and the production of formic acid and consumption of periodate determined (Methods 3 and 4 p. 17). The results are expressed as moles per mole of maltose.

Results.

Wt. of maltose = 212 mg.
Sodium hydroxide solution = 0·01185 N
Iodine solution = 0·100 N.

Formic acid release

TABLE 5

Time (hr.)	Titre (ml.)	Blank (ml.)	Corrected Titre	Formic Acid (moles) (a)	(b)
2	3·83	0·00	3·83	1·46	1·46
4	4·48	0·08	4·40	1·71	1·68
25	5·95	0·49	5·46	2·27	2·08
48	6·96	0·64	6·32	2·66	2·42
72	8·12	0·39	7·73	3·10	2·95

(a) calculated from the titre after correction for the original (2 hr.) value of the blank.

(b) calculated from the titre after correction for the value of the blank at time of analysis.

Periodate uptake

TABLE 6

Time (hr.)	Titre (ml.)	C.T. (a) (ml.)	Periodate uptake (moles)
2	3·53	2·14	3·45
25	3·97	2·58	4·16
48	4·02	2·63	4·24
72	4·14	2·75	4·44

A second experiment gave results as shown in Table 7.

TABLE 7

Time (hr.)	Formic Acid Release (moles)		Periodate uptake (moles)
	(a)	(b)	(a)
2	1.51	1·51	4·14
25	2·50	2·48	4·64
48	2·61	2·56	4·82
72	2·93	2·92	5·06

In additional experiments the formic acid release (a), after 25 hours, varied from 2·2 - 2·5 moles per mole of maltose, the periodate uptake (a) being 4·2 - 4·5 moles.

At 18 - 24 hours a white precipitate formed in the reagent blank

and, to a much lesser extent, in the maltose oxidation mixture.

(2) Examination of the reagent blank.

The following solutions were prepared and stored in the dark at 2°C.

A. 20 ml. 0·28 M- sodium metaperiodate plus 20 ml. distilled water.

B. 20 ml. 0·28 M- sodium metaperiodate plus 20 ml. 3% sodium chloride solution.

C. 20 ml. 0·28 M- sodium metaperiodate plus 20 ml. 4 4% sodium nitrate.

Aliquots (5 ml.) were removed at intervals and the pH. measured using a glass electrode and a Pye Universal pH. meter. Portions (1 ml.) were then added to ethylene glycol (0·5 ml.) and titrated against 0·01177 N - sodium hydroxide solution.

Results.

TABLE 8

Time (days)	A pH.	A Titre	B pH.	B Titre	C pH.	C Titre
2	4·5	0·04	4·0	0·07	4·1	0·05
5	4·1	0·04	3·3	0·50	3·3	0·28
10	4·1	0·04	3·0	0·56	3·1	0·41

After 2 days no precipitation was observed in any solution. After 4 - 5 days a light precipitation occurred in B and C but not in A.

(3) Oxidation in aqueous solution.

A solution containing 0·37 M- sodium metaperiodate (10 ml.) and maltose (200 mg., 10 ml.) was placed, together with a reagent blank, in the dark at 2°C. Aliquots (1 ml.) were removed and analysed as before. The reagent blank was, in this case, stable, sodium hydroxide and iodine titres both remaining constant.

Results are given for the above oxidation and also for a duplicate experiment in which the formaldehyde release was estimated (Method 5 p. ( ) using an aliquot (2 ml.) of a solution prepared by diluting the maltose oxidation mixture (1 ml.) with water (24 ml.) at 2°C.

Results.

TABLE 9

Time (hrs.)	Formic Acid Release		Periodate Uptake		Formaldehyde Release
	(1)	(2)	(1)	(2)	(2)
4	1·7	1·5	-	3·8	-
25	2·4	2·2	4·4	4·2	0·29
72	2·6	2·5	4·6	4·4	0·45
144	-	2·9	-	4·9	0·95

In further experiments the formic acid release, after 25 hours, was 2·2 - 2·4 moles, the periodate uptake being 4·2 - 4·5 moles. After 72 hours these values were 2·6 - 2·8 moles and 4·6 - 4·8 moles respectively.

(4) Continued liberation of formic acid after addition of ethylene glycol.

Aliquots (1 ml.) were removed after 48 hours from one of the above oxidation mixtures. After destruction of periodate with ethylene glycol (1 ml.) these aliquots were titrated, in a stream of carbon dioxide-free air, with sodium hydroxide using methyl red indicator. The flasks containing the neutralised solutions were then firmly stoppered and set aside in the dark at room temperature. Additional sodium hydroxide, to restore neutrality, was added at intervals.

Results.

TABLE 10

Time after addition of ethylene glycol	20 min.	2 hr.	3 hr.	24 hr.
Corrected Titre (mls.)	6·70	7·12	7·23	7·61
Formic Acid (moles).	2·56	2·74	2·81	2·98

In a second such experiment the titre increased, on standing at room temperature for 24 hours, from 6·18 to 7·00 ml. (2·39 to 2·71 moles). The titre of a sample removed at 72 hours from this oxidation mixture had a corrected titre of 6·73 mls. (2·61 moles).

(5) Hydrolysis of formyl esters.

The following solutions were prepared and placed in the dark at 2°C.:-

A. 0·37 M - Sodium Metaperiodate (10 ml.), ethyl formate (1 ml.), 0·1 N - formic acid (1 ml.), water (8 ml.).

B. 0·37 M - Sodium Metaperiodate (10 ml.), propyl formate (1 ml.), 0·1 N - formic acid (1 ml.), water (8 ml.).

C. 0·37 M - Sodium Metaperiodate (10 ml.), 0·1 N - formic acid (1 ml.), water (9 ml.). (REAGENT BLANK).

Aliquots (1 ml.) were withdrawn at intervals into ethylene glycol and titrated with sodium hydroxide.

Results.

TABLE 11

Time (days)	Corrected Titre (ml.)	
	A	B
1	1·52	1·45
2	2·30	1·83
6	8·72	5·01
9	9·58	5·82
12	15·50	7·50
15	20·30	11·32

The titres for C remained constant.

Potassium Metaperiodate Oxidation of Glycogen (39) (42).

Glycogen (200 - 300 mg.) was dissolved in 3% potassium chloride solution (100 ml.) in a brown glass bottle; 0·37M - sodium metaperiodate solution was then added (15 ml.). A reagent blank was also prepared and both solutions were placed on a mechanical shaker. Aliquots (10 ml.) were removed at intervals, care being taken to include none of the solid potassium periodate (which settled readily on standing), and the formic acid concentration determined by titration (Method 4), corrections being made for the reagent blank and for any initial acidity of a zero time sample.

Results.

TABLE 12

Glycogen Sample	Time (hrs.)	Formic acid (%)	C.L.
Rabbit liver V	240	2·27	12·5
	390	2·42	11·8
Rabbit liver X	300	2·36	12·0
	500	2·36	12·0
Rabbit liver XX	300	2·20	12·9
	500	2·30	12·3

TABLE 12

(Contd.)

Glycogen sample	Time (hrs.)	Formic acid (%)	C.L.
Tetrahymena pyriformis II	240	1·97	14·5
	390	2·08	13·7
Trichomonas gallinae II	240	2·11	12·9
	390	2·09	13·0

Sodium Metaperiodate Oxidation of Glycogen.

0·37 M - Sodium metaperiodate solution (2 ml.) was added to a solution of glycogen in water (100 mg., 23 ml.). This solution, together with a reagent blank, was placed in the dark at 2°C. Aliquots (5 ml.) were withdrawn, at intervals, into ethylene glycol (0·5 ml.) and the formic acid production determined as before, corrections again being made for the reagent blank and for any initial acidity of zero time samples.

Results are shown in Table 13.

TABLE 13

Glycogen sample	Time (days)	Formic Acid (%)	C.L. (glucose residues)
Rabbit liver V	7	2·16	13·2
	9	2·27	12·5
	11	2·28	12·4
Rabbit liver X	11	1·38	21
	13	1·41	20
Rabbit liver I	11	2·20	12·9
	13	2·10	13·5
Rabbit liver XX	11	2·31	12·3
	13	2·32	12·2
Rabbit liver XXI	8	2·22	12·8
	12	2·22	12·8
Trichomonas gallinae II	8	2·25	12·6
	12	2·25	12·6
Tetrahymena pyriformis II	8	2·22	12·8
	12	2·26	12·5
Ascaris lumbricoides	8	2·45	11·6
	12	2·47	11·5
Human muscle	12	2·50	11·4
	14	2·50	11·4
Cat liver VI	7	0·84	34
	9	0·90	32
Amylopectin samples			
Rice	8	1·48	18·8
	10	1·48	18·8
Waxy maizestarch II	8	1·36	20·8
	10	1·38	20·5
Waxy maize starch IV	8	1·28	22·1
	10	1·30	21·8

Repeated Oxidation of Cat liver VI and Rabbit liver X Glycogens.

Cat liver VI and rabbit liver X glycogens were oxidised under the same conditions, 4 ml. 0·37 M- sodium metaperiodate being added to 200 mg. glycogen in a total volume of 25 ml. Formic acid production was measured as before; the periodate uptake was also determined by withdrawing aliquots

(1 ml.) into standard arsenite solution (Method 3, p. 17).

Results.

TABLE 14

Sample	Time (days)	Periodate Uptake	Formic Acid (%)	C.L.
Rabbit liver X	4	1·03	-	-
	7	1·04	1·70	16·6
	14	1·07	2·25	12·6
	21	1·11	2·38	11·9
	28	1·20	2·62	10·8
Cat liver VI	4	0·92	-	-
	7	0·99	1·50	18·8
	14	1·02	-	-
	28	1·06	2·09	13·6

Oxidation at room-temperature with increased periodate concentration.

Glycogen (200 mg.) was oxidised in the dark at room temperature with 0·37 M - sodium metaperiodate (5·5 ml.) in a total volume of 20 mls. Periodate uptake and formic acid release were determined as before. Results are given in Table 15.

TABLE 15

Sample	Time (days)	Periodate Uptake (moles)	Formic Acid (%)	C.L.
Rabbit liver X	6	1·06	2·12	13·4
	8	1·08	2·39	11·9
	10	1·07	2·39	11·9
	15	-	2·92	9·8
Cat liverVI	6	-	1·90	17·6
	9	1·04	2·05	13·8
	11	-	2·22	12·6
	13	1·09	2·37	11·9
	17	1·09	2·37	11·9

SECTION IV

THE ABSORPTION SPECTRA OF THE IODINE COMPLEXES OF AMYLOPECTIN - GLYCOGEN TYPE POLYSACCHARIDES.

I. INTRODUCTION

It is well known that iodine forms an intensely blue coloured complex with the amylose component of starch. This has been used as a qualitative test for starch, and forms the basis of methods of determination of the amylose content of starch samples by colorimetric estimation (162) and by potentiometric titration (61). Amylopectin and glycogen stain much less intensely than amylose and form purple brown to yellow brown complexes.

The action of branching or debranching enzymes, which synthesise or hydrolyse α - 1:6 glucosidic inter-chain linkages in starch-type polysaccharides, has been followed by measurement of changes in the iodine staining power of the substrate (62) (63) (64). For example, on incubation of amylose with yeast branching enzyme the wavelength of maximum absorption (λ max.) of the polysaccharide-iodine complex falls from 640 to 520 m.μ. (65). However, although there is some evidence that the λ max. of an amylose-iodine complex is related to the chain length of the amylose (provided that the latter does not exceed ca. 100 glucose residues) (66), information on the relationship between the absorption spectrum of a glycogen- or amylopectin-iodine complex and the degree of branching in the polysaccharide is limited.

An examination of the iodine staining power of glycogen has previously

been made by Schlamowitz (67) who used several samples of rabbit liver glycogen of apparently different chain lengths. The absorption spectrum of the glycogen-iodine complexes were determined in 50% saturated ammonium sulphate solution under conditions in which glycogen was in excess, the ratio (W/W) of glycogen to iodine being 256:1. Schlamowitz found that, under these conditions, the λ max. of the iodine-glycogen complexes was the same (496 m.μ) for each glycogen and was independent of chain length. However, the optical density at the λ max. (O.D. max.) was roughly proportional to the C.L. of the glycogen sample.

A sample of oyster glycogen was also examined. The value of λ max. (360 m.μ) differed markedly from that of the rabbit liver glycogens and there was no proportionality in C.L. and O. D. max. values between the oyster glycogen and the rabbit liver samples. Schlamowitz also found that the absorption spectrum of the iodine complex (in water) of a rabbit liver glycogen of C.L. 23, was very different in both λ max. and O.D. max. from that of an amylopectin which apparently had the same C.L. value. The significance of these results is lessened since C.L. values of the samples were determined by the method of Potter and Hassid (45) which has since been shown to be unreliable. It appears from the above results, however, that absorption spectra of glycogens prepared from the same source (i.e. same biological species) is related to the C.L. of the glycogens, and that such absorption spectra may differ markedly from those of amylopectins. It is possible therefore that (a) there may be a relationship between the absorption spectra of iodine complexes of glycogens of different biological sources and known structural features of the glycogens, and (b) the absorption spectra of amylopectin-iodine complexes may differ in a characteristic manner from those of glycogens.

In the present work the absorption spectra of the iodine complexes of several glycogens and amylopectins of known branching characteristics have been examined. Under the conditions used (glycogen:iodine $^{W}/_{W}$ ratio, 1:2) which are similar to those described by Peat and his co-workers (64) only a few mg. of polysaccharide are required for spectral measurements. Any relationship between the spectrum of the iodine complex and structural features of the polysaccharide would therefore be of especial value in the examination of amylopectin-glycogen type polysaccharides of microbiological origin which are often difficult to obtain in amounts sufficient for examination by standard chemical methods. Several previously described protozoal polysaccharides of the amylopectin-glycogen type have been included in the present investigation.

II. DISCUSSION

The Absorption Spectra of the Iodine Complexes in Aqueous Solution.

The absorption spectra of the iodine complexes of several samples of glycogen, isolated from various sources, were examined in aqueous solution (Table 16). The spectra of glycogens isolated from the protozoa Tetrahymena pyriformis, Trichomonas gallinae and Trichomonas foetus were similar to those of typical animal glycogens (Figs. 5, 6 and 7).

From the collected results (Table 16) it is seen that the λ max. values of the iodine complexes of glycogens are in the region 420 - 470 m.μ. , the maximum optical density (O.D.) values being 0·11 - 0·37 units. Graphical representation of these results shows that there is little or no significant relationship between O.D. max. or λ max. values and the average exterior chain length (E.C.L.), average chain length (C.L.), or average interior chain length (I.C.L.) values of the glycogen samples (Figs. 8, 9).

The spectra of the iodine complexes of several amylopectins, including two of protozoal origin, were then examined (Table 17). These differed sharply from those of the glycogens, the λ max. values of the amylopectin complexes being in the range 510 - 540 m.μ. and having O.D. values 0·45 - 1.22. The absorption spectra of the amylopectin component of Chilomonas paramecium starch and of an amylopectin-type polysaccharide isolated by Dr. A. E. Oxford from the holotrich ciliates of sheeps rumen were similar to those of typical plant amylopectins.

TABLE 16

The Iodine Staining of Glycogen.

Sample	Aqueous Solution		50% Ammonium sulphate solution		C.L.	I.C.L.	E.C.L.	
	λmax. (mμ.)	O.D.	λ max. (mμ.)	O.D.				
Rabbit liver VI	470	0·33	500	1·07	18	5	12	
Rabbit liver XII	-	-	500	1·02	17	6·5	9·5	
Rabbit liver XI	470	0·36	500	0·92	16	-	-	
Trichomonas foetus II	445	0·37	500	1·06	15	2·5	11·5	
Rabbit liver XIII	430	0·25	480	1·01	15	4·5	9·5	
Human liver (storage disease)	-	-	480	0·95	15	4·5	9·5	
Tetrahymena pyriformis II	440	0·26	485	0·87	14	-	-	
Rabbit liver '54	440	0·25	480	0·99	14	3·5	9·5	
Trichomonas gallinae II	440	0·26	480	0·99	13	-	-	
Tetrahymena pyriformis I	445	0·32	465	0·90	13	3·5	8·5	0·006
Rabbit muscle	-	-	490	0·91	13	3·5	8·5	
Lights (commercial sample)	460	0·11	480	1·00	13	3·5	8·5	
Ascaris lumbricoides	435	0·12	480	1·07	12	3	8	0·009
Human muscle	445	0·14	460	0·59	11	3	7	0·009
Oyster	460	0·13	480	1·07	11	-	-	
Mytilus edulis	420	0·16	450	0·77	9	2	6	
Helix pomatia	425	0·12	450	0·88	7	2	4	

TABLE 17

The Iodine Staining of Amylopectin

Amylopectin sample	Aqueous solution		50% Ammonium sulphate solution		C.L.	I.C.L.	E.C.L.
	λmax. (mμ.)	O.D.	λmax. (mμ.)	O.D.			
Waxy sorghum starch I	540	1·06	540	1·16	25	7·5	16·5
Waxy sorghum starch II	535	0·96	565	1·18	25	7	17
Waxy maize starch I	530	1·07	530	1·18	22	6·5	14·5
Waxy maize starch II	530	0·85	550	1·07	21	5	15
Waxy maize starch IV	530	1·08	560	1·59	22	6	15
Chilomonas paramecium	540	1·22	545	1·38	22	5·5	15·5
Holotrich ciliate	530	1·14	530	1·03	22	-	-
Rice	510	0·45	525	0·75	19	-	-

The absorption spectra of the iodine complexes in ammonium sulphate solution

The spectra of the protozoal glycogens in 25% and in 50% saturated ammonium sulphate solution are shown graphically (Figs. 5, 6, 7). As with all glycogens examined, the solutions in 50% ammonium sulphate were turbid and the glycogen-iodine complex precipitated. To minimise the error resulting from such precipitation, the spectra of the solutions were examined

THE ABSORPTION SPECTRUM OF THE IODINE COMPLEX OF TRICHOMONAS ~~FOETUS~~ GALLINAE GLYCOGEN IN (A) WATER, (B) 25% SATURATED AMMONIUM SULPHATE SOLUTION, AND (C) 50% SATURATED AMMONIUM SULPHATE SOLUTION.

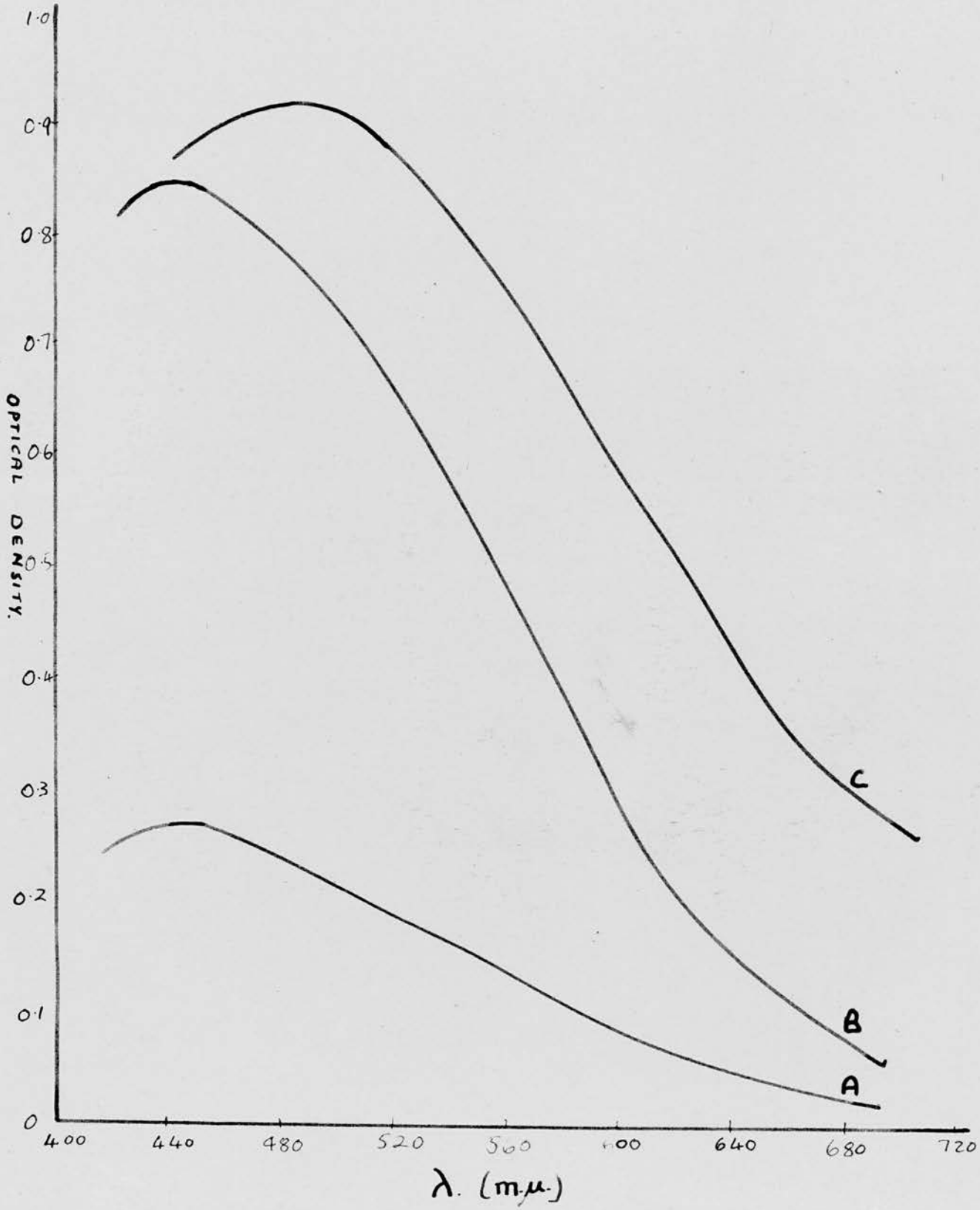


Fig. 5

THE ABSORPTION SPECTRUM OF THE IODINE COMPLEX OF TETRAHYMENA PYRIFORMIS GLYCOGEN IN (A) WATER, (B) 25% SATURATED AMMONIUM SULPHATE SOLUTION, AND (C) 50% SATURATED AMMONIUM SULPHATE SOLUTION.

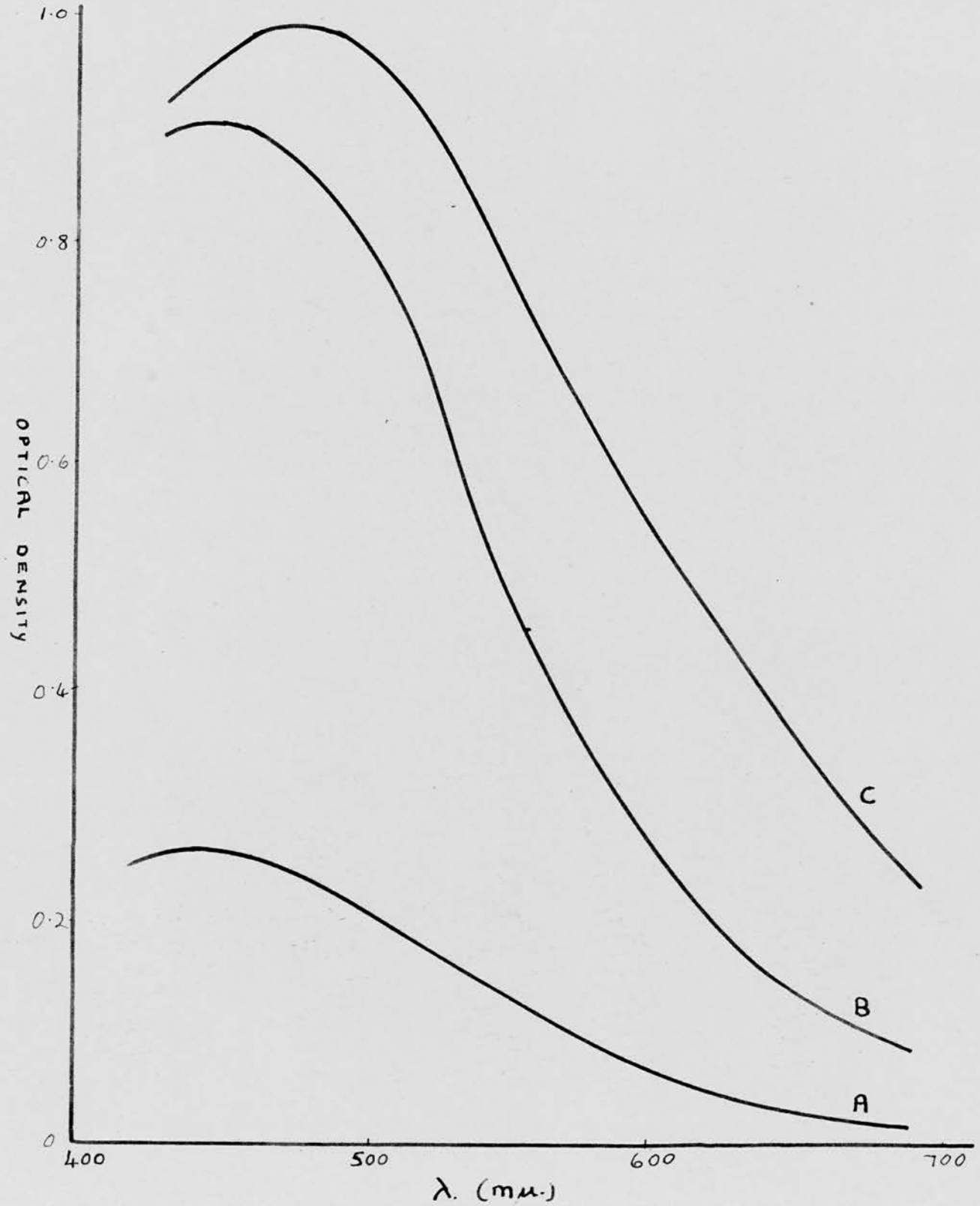


Fig. 6

THE ABSORPTION SPECTRUM OF THE IODINE COMPLEX OF TRICHOMONAS FOETUS GLYCOGEN IN (A) WATER, (B) 25% SATURATED AMMONIUM SULPHATE SOLUTION, AND (C) 50% SATURATED AMMONIUM SULPHATE SOLUTION.

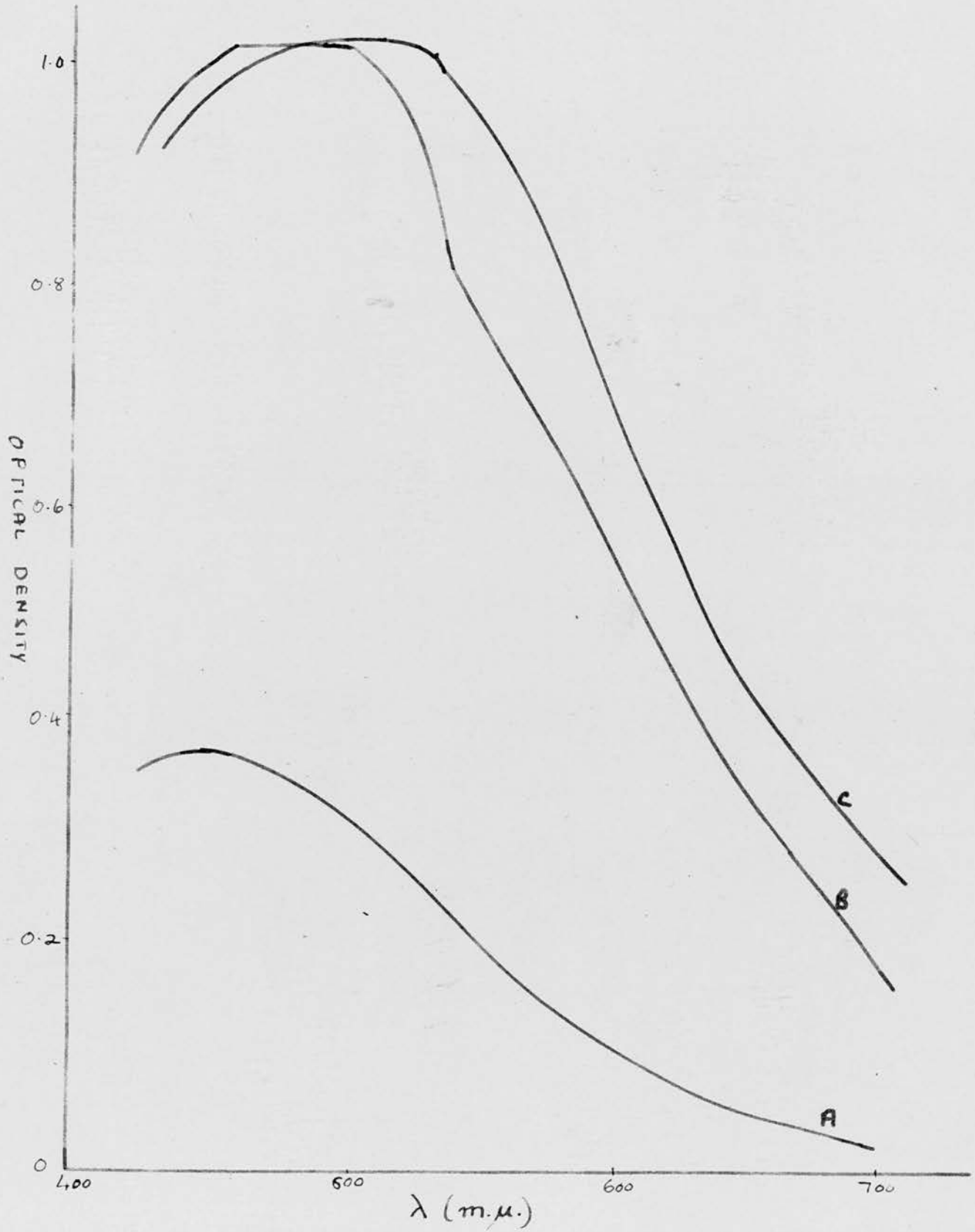


Fig 7.

THE RELATIONSHIP BETWEEN THE BRANCHING CHARACTERISTICS
AND THE ABSORPTION SPECTRA OF THE
IODINE COMPLEXES OF BRANCHED α- 1:4 - GLUCOSANS ... (1)

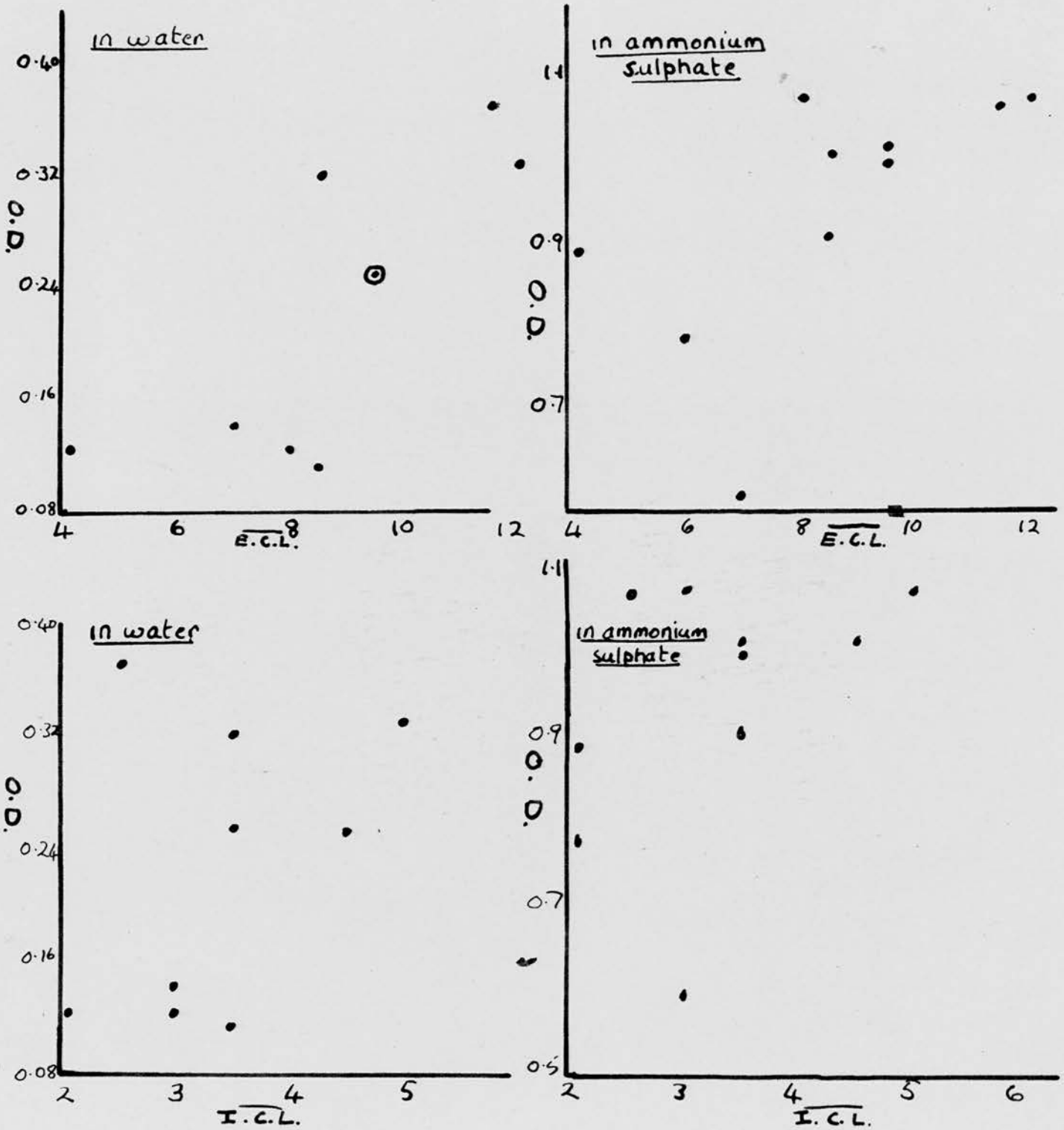
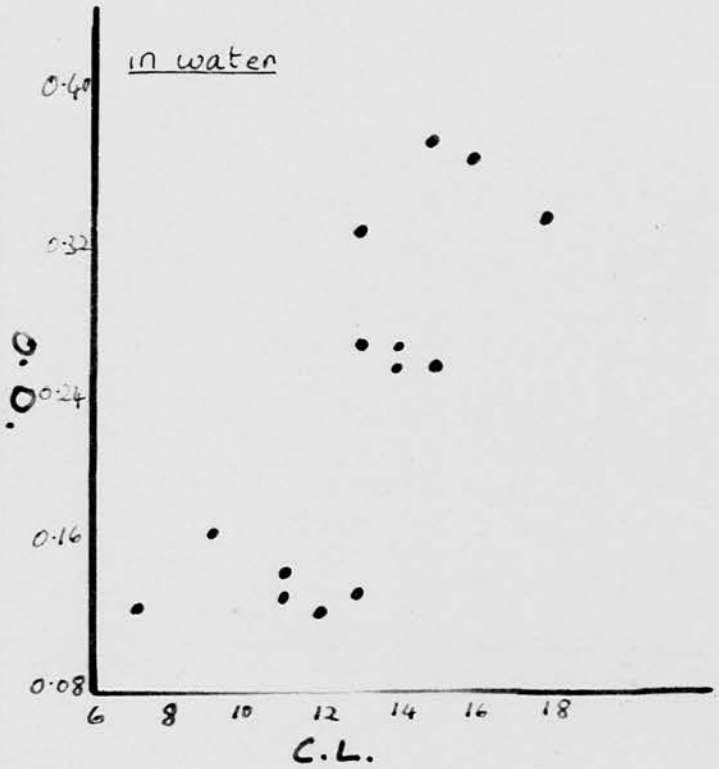
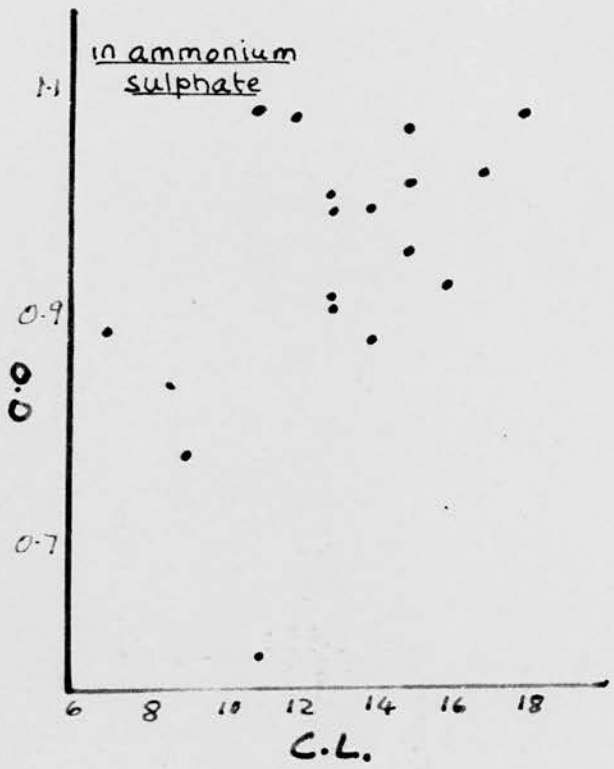
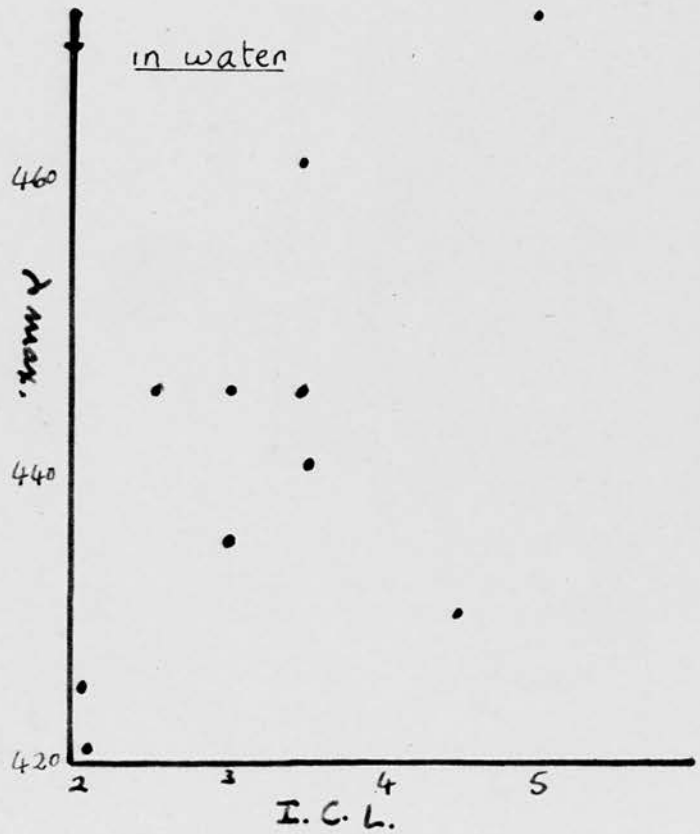


Fig 8

THE RELATIONSHIP BETWEEN THE BRANCHING CHARACTERISTICS
AND THE ABSORPTION SPECTRA OF THE
IODINE COMPLEXES OF BRANCHED α - 1:4 - GLUCOSANS ... (2)







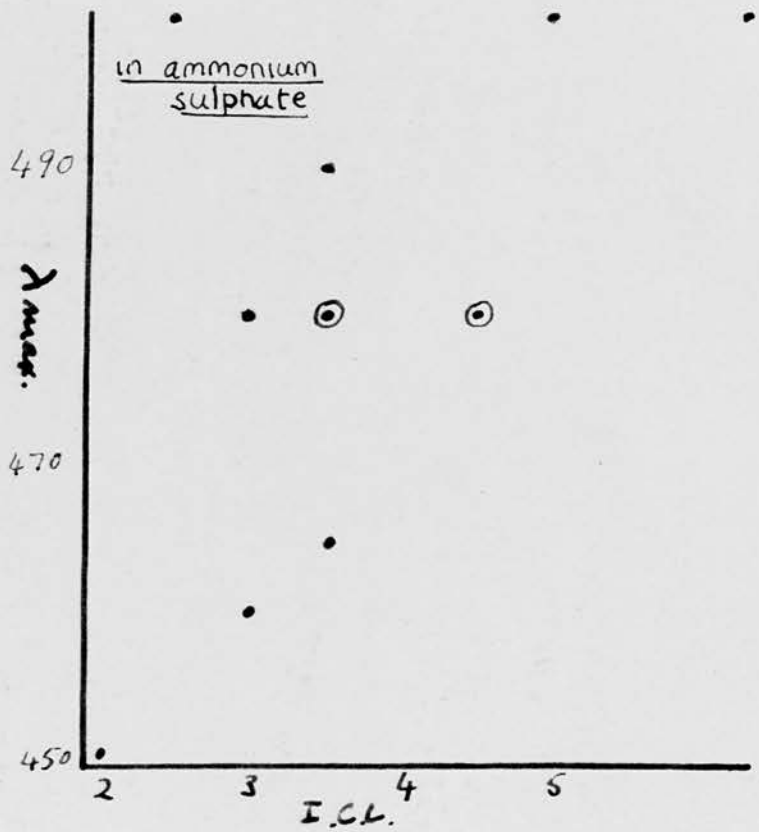


Fig 9.

THE RELATIONSHIP BETWEEN THE BRANCHING CHARACTERISTICS
AND THE ABSORPTION SPECTRA OF THE
IODINE COMPLEXES OF BRANCHED α - 1:4 - GLUCOSANS ... (3)

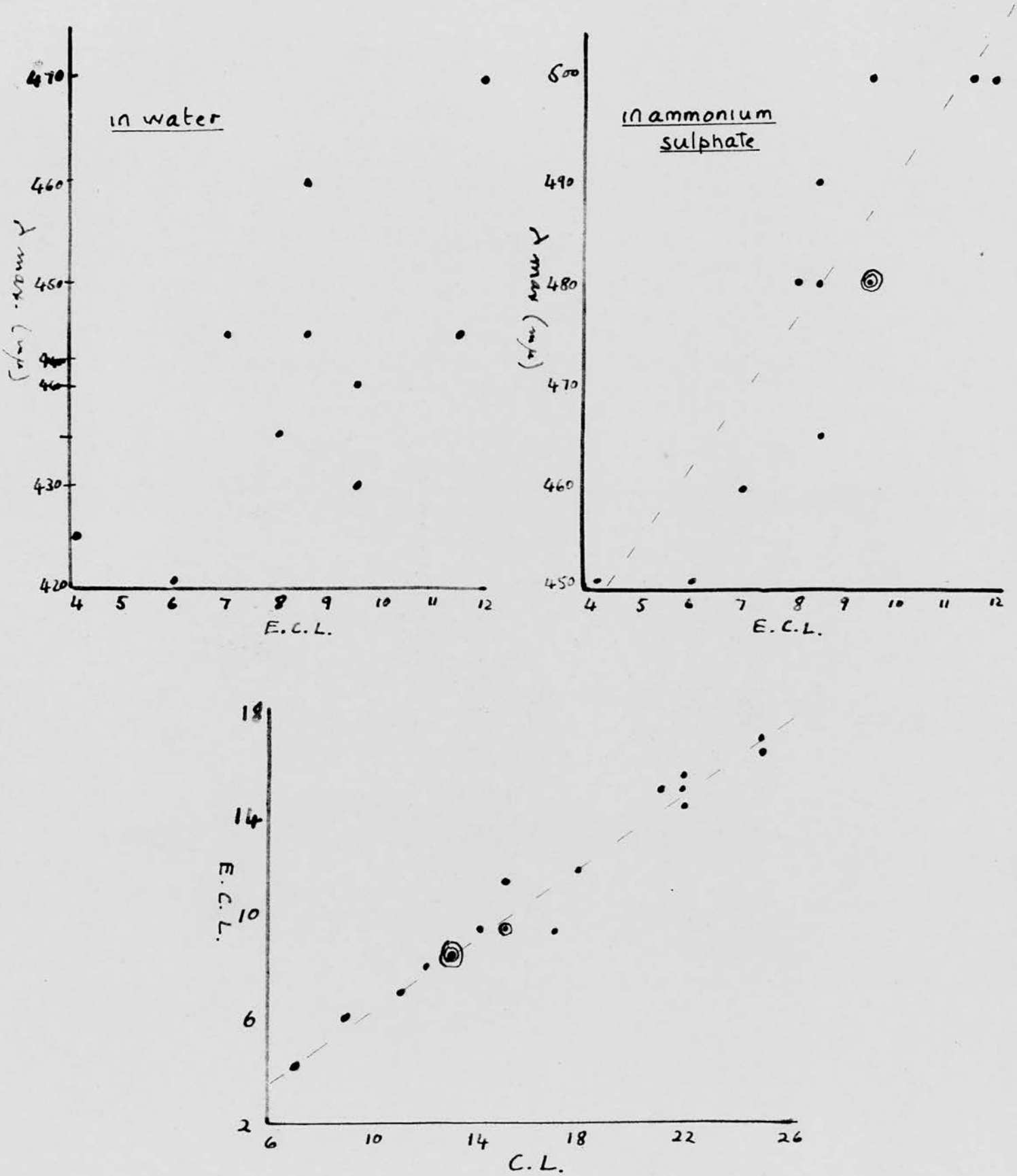
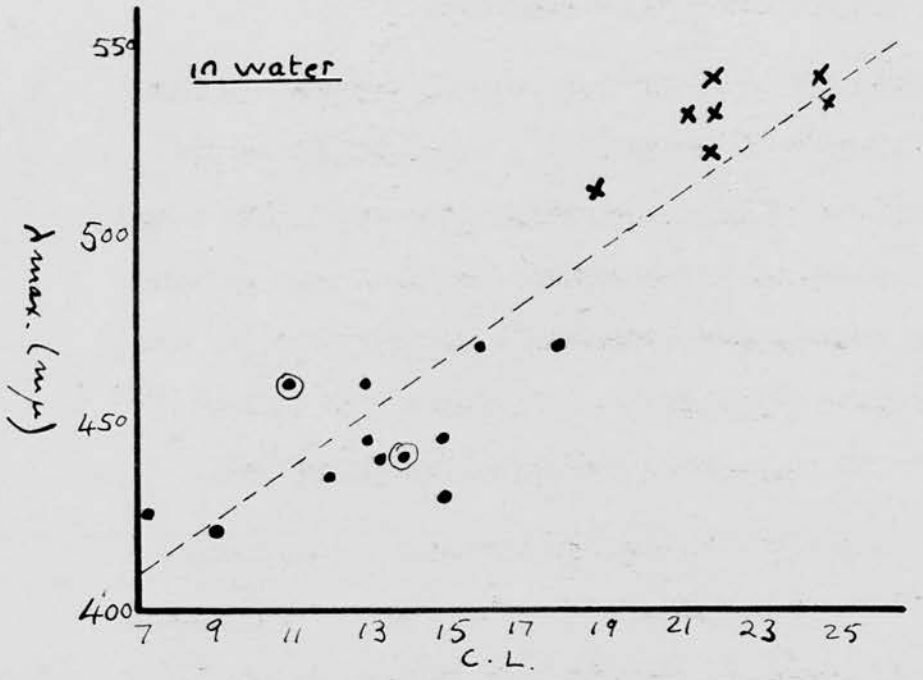
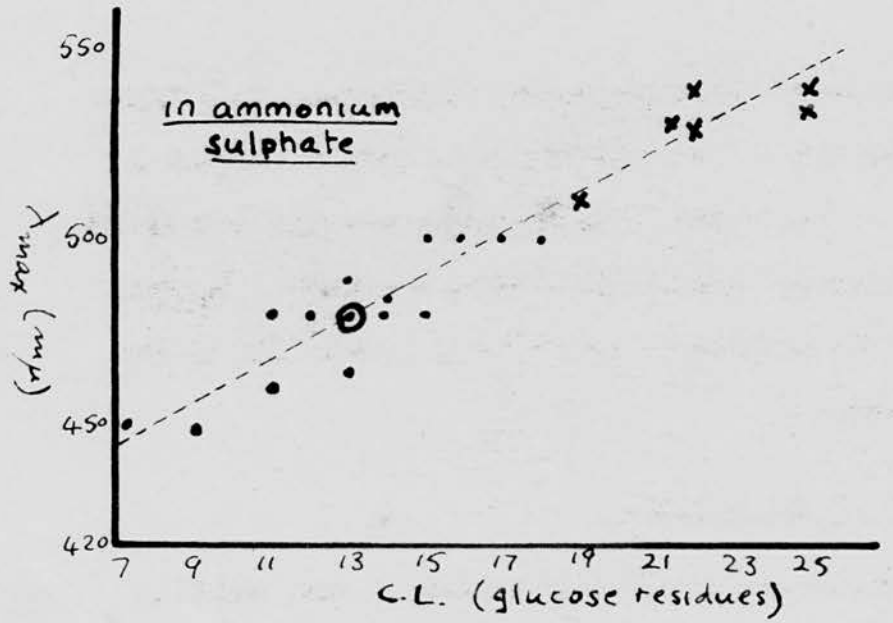


Fig. 10

THE RELATIONSHIP BETWEEN THE BRANCHING CHARACTERISTICS AND THE ABSORPTION SPECTRA OF THE IODINE COMPLEXES OF BRANCHED α - 1:4 - GLUCOSANS ... (4)





• denotes value for glycogen

X denotes value for amylopectin

Fig 11.

immediately after the addition of iodine. Values of λ max. of such solutions were reproducible ($\pm$ 5 m.μ.) although O.D. max. values decreased slowly with time. In water and in 25% ammonium sulphate solution the glycogen-iodine complexes formed clear stable solutions.

Collected results for all protozoal and animal glycogens are shown in Table 16. From these results it is seen that λ max. values in 50% saturated ammonium sulphate are in the region of 450 - 500 m.μ., O.D. values being 0·59 - 1·07 units. Graphical representation of these results shows that there is little or no relationship between O.D. values and C.L., E.C.L. or I.C.L. values of the samples (Figs. 8, 9). In view of the lack of reproducibility of the O.D. values, such a finding is not unexpected.

There is, however, a marked relationship between λ max. and the C.L. and E.C.L. values of the samples (Figs. 10, 11). The λ max. values of the amylopectins in water appear to bear the same relationship to C.L. and E.C.L. as do the λ max. values of the glycogens in 50% saturated ammonium sulphate.

The absorption spectra of the amylopectins in 50% ammonium sulphate solution were then examined (Table 17). It was found that the effect of ammonium sulphate on the λ max. values of amylopectins was much less marked than with glycogens, many of the λ max. values being unaltered. As with glycogens, O.D. values of the amylopectin complexes were increased by the presence of ammonium sulphate.

Mathematical consideration of the results.

In view of the apparent proportionality between λ max. and C.L. and E.C.L., the various results were examined mathematically, best straight

lines being determined by the method of least squares. The standard deviations (σ) were then calculated and since, as can be shown statistically, 95% of the results are expected to lie within $1 \cdot 645 \times \sigma$, any values outside this range were taken to represent significant differences. The factor $1 \cdot 645 \times \sigma$, is referred to hereafter as the "maximum probable error (M.P.E.)".

Using the relationships thus calculated for the glycogen samples, theoretical values of λ max. for the amylopectin complexes were calculated from C.L. or E.C.L. values of the amylopectin (Table 18). The deviation of such calculated results from the observed values of λ max. of amylopectin complexes gives a measure of the correlation between λ max. values of amylopectin and those of glycogen.

(1) λ max. versus C.L. (aqueous solution).

Best straight line:-

$$\lambda \text{ max.} = 3 \cdot 43 \times \text{C.L.} + 401 \quad \ldots\ldots \text{ equation (1)}$$

Standard deviation, = 11·5

Maximum probable error= 18·9 m.μ. or 5·5 - C.L. units.

The differences between λ max. values for amylopectins calculated, using equation (1), from the C.L. values of the amylopectins, and the observed values of λ max. of the amylopectin complexes in aqueous solution are as shown in Table 18. The mean difference, 53, is significantly larger than the maximum probable error of 18·9, showing that there is a significant difference between the spectra of the complexes of glycogen in aqueous solution and those of amylopectin in aqueous solution.

TABLE 18

A Comparison of Observed and Calculated λ max. Values for Several Amylopectins

Amylopectin sample	λ max. observed	λ max. value calculated from equation				λobs. $-\lambda$c. (1)	λobs. $-\lambda$c. (2)	λobs. $-\lambda$c. (3)	λobs. $-\lambda$c (4)
		(1)	(2)	(3)	(4)				
Waxy sorghum starch I	540	487	477	541	537	53	63	1	3
Waxy sorghum starch II	535	487	479	541	541	48	56	6	6
Waxy maize starch I	530	477	468	525	522	53	62	5	8
Waxy maize starch II	530	473	470	520	526	57	60	10	4
Waxy maize starch IV	530	477	470	525	526	53	60	5	4
Chilomonas paramecium	540	477	472	525	530	63	68	15	10
Holotrich ciliate	530	477	-	525	-	53	-	5	-
Rice	510	466	-	510	-	44	-	0	-
					Mean Deviation	53	62	6	5

(2) λ max. versus E.C.L. (aqueous solution).

Best straight line:-

$$\lambda max. = 4{\cdot}28 \times E.C.L. + 406 \quad \ldots\ldots \text{ equation (2).}$$

$$\sigma = 10{\cdot}8 \text{ m}\mu.$$

$$M.P.E. = 17{\cdot}8 \text{ m}\mu. \text{ or } 4{\cdot}2 \text{ E.C.L. units.}$$

Mean deviation between the calculated (equation 2) and observed values of λ max. for amylopectin is 62 mμ., again showing a significant difference between the spectra of the aqueous complexes of glycogen and amylopectin.

(3) λ max. versus C.L. (50% saturated ammonium sulphate).

Best straight line:-

$$\lambda max. = 5{\cdot}19 \times C.L. + 411 \quad \ldots\ldots \text{ equation (3)}$$

$$\sigma = 7{\cdot}68 \text{ m}\mu.$$

$$M.P.E. = 12{\cdot}6 \text{ m}\mu. \text{ or } 2{\cdot}4 \text{ C.L. units.}$$

The mean deviation between the calculated (equation 3) values of λ max. and those observed in aqueous solution was 6 mμ. showing that there is a significant relationship between the spectra of glycogen complexes in 50% ammonium sulphate, and those of amylopectin in water.

(4) λ max. versus E.C.L. (in 50% saturated ammonium sulphate solution).

Best straight line:-

$$\lambda max. = 7{\cdot}34 \times E.C.L. + 416 \quad \ldots\ldots \text{ equation (4)}$$

$$\sigma = 8{\cdot}16 \text{ m}\mu.$$

$$M.P.E. = 13{\cdot}4 \text{ m}\mu. \text{ or } 1{\cdot}83 \text{ E.C.L. units.}$$

The mean deviation between calculated values of λ max. and those

observed for the amylopectin complex in aqueous solution was 5 m.μ. again indicating the existance of a significant relationship between the spectra of the iodine complexes of glycogens in 50% ammonium sulphate and of the amylopectin complexes in aqueous solution.

The best straight line relationships between E.C.L. and C.L. values of the samples, and the λ max. values of the glycogen complexes in 50% ammonium sulphate and of the amylopectin complexes in aqueous solution were therefore calculated.

(5) λ max. versus C.L.

Best straight line:-

$$\lambda \text{ max.} = 5{\cdot}51 \times \text{C.L.} + 408 \quad \ldots\ldots \text{ equation (5)}$$

$$\sigma = 7{\cdot}3 \text{ m.}\mu.$$

$$\text{M.P.E.} = 12 \text{ m.}\mu. \text{ or } 2{\cdot}2 \text{ C.L. units.}$$

(6) λ max. versus E.C.L.

Best straight line:-

$$\lambda \text{ max.} = 7{\cdot}87 \times \text{E.C.L.} + 411 \quad \ldots\ldots \text{ equation (6)}$$

$$\sigma = 8{\cdot}6 \text{ m.}\mu.$$

$$\text{M.P.E.} = 14 \text{ m.}\mu. \text{ or } 1{\cdot}8 \text{ units.}$$

The relationship between λ max. and both C.L. and E.C.L. values indicates that there is a relationship between the C.L. and the E.C.L. values of the samples examined. Graphical representation (Fig. 10) showed that this suggestion was correct, the best straight line (calculated as before) being:-

$$\text{C.L.} = 1{\cdot}35 \times \text{E.C.L.} + 1{\cdot}7 \quad \ldots\ldots \text{ equation (7)}$$

$$\sigma = 1{\cdot}3 \text{ C.L. units.}$$

$$\text{M.P.E.} = 2{\cdot}2 \text{ C.L. units or } 1{\cdot}6 \text{ E.C.L. units.}$$

From these results it is apparent that, in aqueous solution, (a) there is little relationship between C.L. or E.C.L. values and the λ max. values of the iodine complexes of glycogen, and that (b) the spectra of amylopectin-iodine complexes are significantly different from those of glycogen.

In 50% ammonium sulphate solution, however, there is a significant proportionality between λ max. values of the iodine complexes of glycogen and the C.L. and E.C.L. values of the polysaccharide samples. Furthermore, there appears to be a significant relationship between such values and those of amylopectin in aqueous solution.

INTERPRETATION OF RESULTS

Schlamowitz (67) has suggested that the spectrum of the polysaccharide-iodine complex is essentially a spectrum of the free iodine molecule, and that the changes produced by the polysaccharide on the spectrum of iodine in water reflect alterations in the structure of the iodine molecule which are induced by the environment into which it is now absorbed.

The iodine molecule may be considered to be a hybrid, the wave function of whose valence bond may be expressed as the contribution of at least three hypothetical structures, the non-polarised electrically symmetrical form I - I, and two polarised forms $I^+ - I'$ and $I' - I^+$, in equilibrium (68) (69). Since the valency electrons are those most apt to influence light absorption, the relative contribution made by the various forms to the actual structure determines the nature of the absorption spectrum of iodine. Electron donor groups (e.g. oxygen, sulphur, basic nitrogen) may co-ordinate with the polarised forms and stabilise them; the resultant displacement of the equilibrium among the three forms would enhance the relative contribution of the polarised forms to the actual

state of the iodine molecule, and a change in the absorption spectrum would occur. Fairbrother (69) has shown that such polarisation of iodine does occur in media possessing electron donor properties and that it is accompanied by a displacement of the absorption peak towards the shorter wavelengths, the degree of displacement being proportional to the electron donor capacity of the medium. This displacement towards shorter wavelengths may be explained by a consideration of the nature of the hybrid forms. When the iodine is free, the non-polarised of the three extreme contributing forms predominates and the energy displacement of the valency electrons from the ground state is minimum. As the contribution of the polarised forms increases, there is an increase in the potential energy of the valency electrons above the ground state. This energy displacement may be equated with $h\nu$, where h is Plancks constant and ν the frequency of absorbed light. The greater the energy displacement, i.e. polarisation by electron donors, the greater the displacement of light absorption towards shorter wavelengths (higher frequencies). Conversely, in the absence of electron donors, when polarisation is minimum, the absorption of light by iodine should be greatest in the red region of the spectrum, and the iodine should appear blue or violet. Schlamowitz considers that the blue or violet colour shown by iodine solutions in the presence of starch-type polysaccharides is due to the provision, by the polysaccharide, of an environment conducive to the existence of the iodine molecule in its free, predominantly non-polar form. This is in accord with the view that in linear polysaccharides such as amylose, and in the long outer chains of amylopectin, the glucose units are arranged in coils or 'helices'. The iodine may be absorbed in these helices which, since the hydroxyl groups are oriented outwards from the perimeter, offer an environment for absorbed iodine which is relatively free of electron donor

groups. As the number of turns in a helix is increased there is a corresponding increase in the hydrophobic nature of its core, and hence the occulusion of water, which acts as an electron donor, is decreased. Consequently, with increasing chain length (i.e. helix length) of the polysaccharide, or unbranched portion thereof, not only should there be an increase in the amount of iodine absorbed but also the state of the adsorbed iodine should be represented more and more by the free, predominantly non-polarised form of the molecule. If, therefore, the outer chains of glycogen and amylopectin are in the form of helices into which the iodine is adsorbed, the λ max. and O.D. max. of the absorption spectrum of the iodine complex should be proportional to the average exterior chain length (E.C.L.).

The present results show that, in 50% ammonium sulphate solution, there is a significant relationship between the λ max. of the iodine complex of an amylopectin or glycogen and its E.C.L. value. The experimental observation that λ max. is also related to C.L. may be explained by the fact that, in the samples examined, C.L. values were proportional to E.C.L. values. O.D. values showed little proportionality to C.L. or E.C.L. values. This is ascribed, in part, to the experimental errors involved in the determination of O.D.

There is, however, little relationship between λ max. values of glycogen complexes in water and the E.C.L. and C.L. values of the glycogen sample. Furthermore, λ max. and O.D. max. values of amylopectin in water are significantly higher than those of comparable glycogens. This indicates that in aqueous solution glycogen is less able to absorb iodine that is amylopectin (cf. rabbit liver VI, glycogen; C.L., 18, λ max., 470 m.μ., O.D., 0·33 : rice amylopectin; C.L. 19, λ max. 510 m.μ., O.D., 0·49).

This supports the report of Schlamowitz that an amylopectin absorbed much more iodine than a glycogen of similar C.L.

The absorption of iodine by glycogen in 50% saturated ammonium sulphate solution is comparable to that of amylopectin in water. Schlamowitz has suggested that ammonium sulphate has the effect of depleting the core of the helix of occluded water thereby increasing the amount of iodine absorbed (and hence the O.D.); the diminished electron donor capacity of the environment is then reflected in the increased λ max. values. This can explain, in part, the effect of ammonium sulphate on the absorption spectrum of a glycogen-iodine complex. Since ammonium sulphate has little or no effect on the λ max. of amylopectin complexes, it is necessary to postulate that the helices in amylopectin are sufficiently hydrophobic to be free from occluded water removable by the dehydrating effect of ammonium sulphate, the increases in O.D. being largely due to the turbidity caused by the precipitation of the amylopectin-iodine complex.

An alternative possibility is that the helices in glycogen are not so accessible to iodine as those in amylopectin. It has been shown that the glycogen molecule is much more compact than that of amylopectin, and it is possible that only those chains on the exterior of the molecule are accessible to any appreciable amount of iodine. On this basis, however, it would be expected that, unless the chains on the exterior of the molecule were of much less than average length, the λ max. values, which are dependant on the electron donor capacity of the environment, should be proportional to E.C.L. and comparable to those of amylopectin, O.D. values only, being significantly different. It is difficult on the basis of accessibility of the helix, to understand the effect of ammonium sulphate,

and Schlamowitz's suggestion therefore appears the more probable.

Despite the observed relationship, the absorption spectrum of iodine complexes of amylopectins and glycogens may be influenced to some extent by factors other than E.C.L. values. Some iodine may be adsorbed onto the interior chains (especially with amylopectin), molecular shape may have some effect, and, since both λ max. and O.D. max. are dependant on the length of the helix, λ max. values obtained from samples of glycogen of identical E.C.L. values may vary slightly depending on the distribution of the lengths of the external chains around the average value (E.C.L.).

Conclusions

1. The absorption spectra of glycogen-iodine complexes in water are significantly different from those of amylopectin, the range of values being (a) with glycogen, λ max., 420 - 470 mμ., O.D., 0·11 - 0·37, and (b) with amylopectin, λ max., 510 - 540 mμ., O.D., 0·45 - 1·22.

2. In the presence of 50% saturated ammonium sulphate, λ max. and O.D. max. values of the spectra of glycogen iodine complexes are increased, the range of values being, λ max., 450 - 500 mμ., O.D., 0·59 - 1·07. Under these conditions the spectra of amylopectin-iodine complexes are similarly affected, although to a lesser extent.

3. Values of λ max. of glycogen complexes in 50% ammonium sulphate solution and of amylopectin complexes in aqueous solution are related and are proportional to the C.L. and E.C.L. values of the polysaccharides. It is suggested that this technique may be advantageously used in the micro-examination of amylopectin-glycogen type polysaccharides.

III EXPERIMENTAL

Examination of the Spectra in Aqueous Solution.

Glycogen (25·0 m.g.) was dissolved in water and the solution diluted to 50 ml. in a standard flask. A portion (5 ml.) of this solution was placed in a standard flask (25 ml.). 5 N - Hydrochloric acid (1 drop) and iodine solution (0·2% iodine in 2% potassium iodide; 2·5 ml.) were then added and the mixture diluted to 25 ml. with water. The resulting coloured solution was examined at room temperature (18°C.) against an iodine blank in a Unicam S.P. 500 spectrophotometer, the O.D. of the solution being determined over the range 420 - 700 m.μ. Results of λ max. and O.D. max. values obtained with several amylopectins and glycogens are given in Tables 16 and 17.

Examination of the Spectra in Ammonium Sulphate Solution.

To the standard flask (25 ml.) containing glycogen or amylopectin solution (5 ml., 2·5 m.g.) and hydrochloric acid (1 drop) was added saturated ammonium sulphate solution (12·5 mls.) followed by iodine solution (2·5 ml.). The coloured solution was diluted to the mark and examined as before against ammonium sulphate-iodine blanks. λ max. and O.D. max. values thus obtained for several amylopectins and glycogens are given in Tables 16 and 17.

SECTION V

THE MOLECULAR STRUCTURE OF THE RESERVE POLYSACCHARIDE SYNTHESISED BY CHILOMONAS PARAMECIUM

I. INTRODUCTION.

Starch, the reserve polysaccharide of most green plants, has been detected by iodine staining in several protozoa (2). In 1939 one such starch was isolated from the flagellate Polytoma (the colourless counterpart of Chlamydomonas) by Brèchot (8) who showed that acid hydrolysis of the material gave glucose. Brèchot also observed that the starch was degraded by several enzyme preparations which were also effective on typical plant starches.

The polysaccharide synthesised by the colourless flagellate Polytomella coeca has been thoroughly investigated by Bourne, Stacey and Wilkinson (10) who showed it had a starch-like constitution. Thymol fractionation yielded amylose, and amylopectin type components, the respective amounts present in the whole starch being 13 - 16% and 84 - 87%. The amylose fraction had a blue value (hereafter referred to as B.V.) of 1·13 [potato amylose has B.V., 1·2 - 1·5 (70)] and a β - amylolysis limit of 89%. The amylopectin fraction had B.V., 0·11 [potato amylopectin has B.V., 0·06-0·16 (70)] and a β - amylolysis limit of 48%. The polysaccharide was thus shown to be similar to normal plant starches. Forsyth and Hirst showed that the polysaccharides synthesised by the ciliate Cycloposthium (18) and by the holotrich ciliates present in sheeps rumen (20) were very similar in structure to the amylopectin

fraction of plant starches. No amylose-type component was detected in these two polysaccharides.

In the present work the polysaccharide synthesised by the flagellate Chilomonas paramecium, the colourless counterpart of the photosynthetic Cryptomonas, has been examined by chemical and enzymic methods. This polysaccharide has been examined previously by Hutcheons and his co-workers (9) who found that on boiling the organisms in neutral solution a material which gave a pure blue colour with iodine was extracted. This iodine complex showed maximum absorption at 625 m.μ.; there was no evidence of a second maximum at 540 m.μ. which is the characteristic value of amylopectins. The dissolved material therefore appeared to be an amylose. The insoluble residue gave a reddish-purple colour with iodine and was therefore assumed to be an amylopectin. Heating of the organisms at 100°C. for 1 hour in 0·5 N - sodium hydroxide extracted a material which gave a purple blue colour with iodine (λ max., 550 m.μ.). From these results it appeared that Chilomonas paramecium synthesises a starch-like polysaccharide. Hutcheons estimated that the amylopectin represented more than half the starch present although experimental details were not reported. The present work was undertaken in order to examine in more detail the structure of the starch and its amylose and amylopectin components.

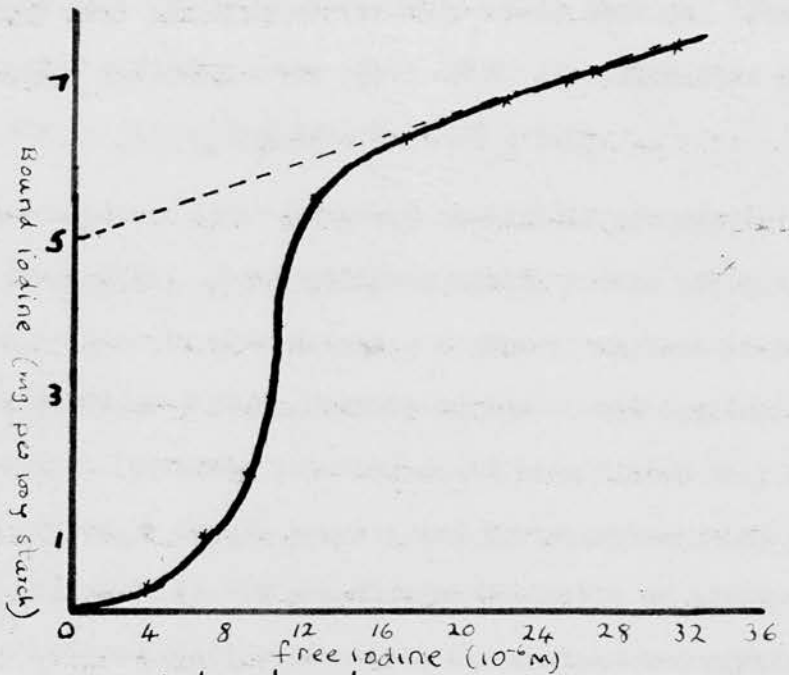
II. DISCUSSION

The present investigation has been carried out on a sample of Chilomonas paramecium starch isolated by Dr. J. F. Ryley by chloral hydrate extraction of the whole cells. This method of extraction had been found satisfactory in the isolation of the previously examined starch type polysaccharides synthesised by Polytomella coeca (10), Cycloposthium (18) and the ciliates of sheeps rumen (20). At the time of extraction (1955) the degradative effect of oxygen on hot starch solutions (71) was not generally appreciated and the extraction of starch was not carried out under anaerobic conditions.

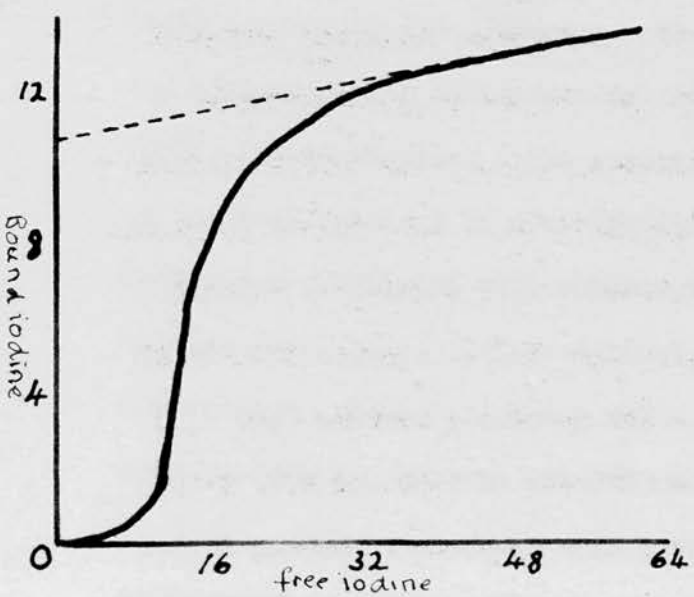
The material thus extracted was a white amorphous powder which gave a blue stain with iodine (B.V., 0·54; λmax., 590 mμ.). Paper chromatographic examination of an acid hydrolysate showed glucose to be the only reducing sugar present. The absence of ketoses was demonstrated by the failure of the hydrolysate to react with the acid resorcinol reagent (72). The material contained 78·4% glucosan, 9·7% ash and 0·94% nitrogen (equivalent to 6% protein). Digestion with barley β - amylase at pH 4·6 resulted in a 75% conversion into maltose. From these preliminary results it is apparent that the polysaccharide is of the starch type. Confirmation of this was obtained by potentiometric iodine titration which has been widely used for the characterisation of starch-type polysaccharides (61). Results obtained by Dr. A. W. Arbuckle were generally similar to those obtained with typical plant starches (Fig. 12), the iodine affinity, expressed as mg. of iodine bound per 100 mg. of starch, being 5·0 (cf. potato starch iodine affinity

POTENTIOMETRIC IODINE TITRATION

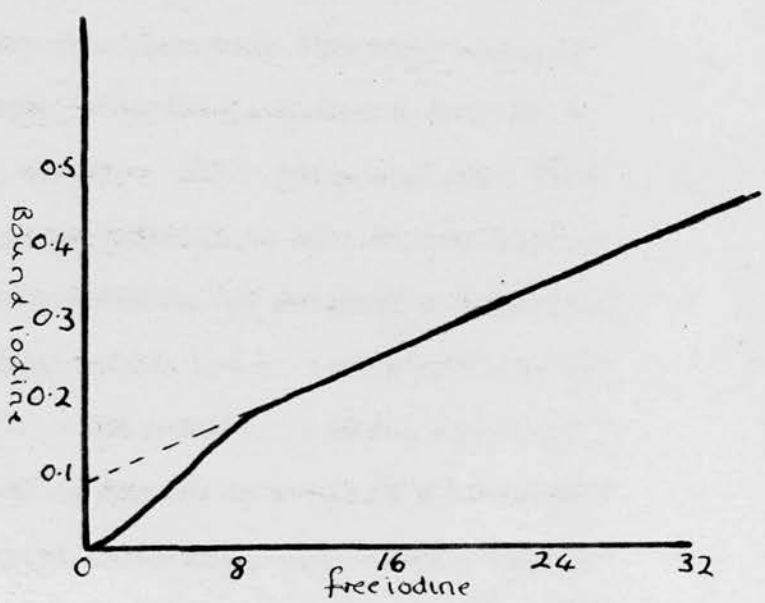
OF CHILOMONAS PARAMECIUM STARCH FRACTIONS



Whole starch



Amylose fraction



Amylopectin fraction

Fig. 12.

ca. 4·0). From a consideration of the iodine affinity or B.V. of the amylose component the percentage of amylose present in the whole starch can be calculated. By comparison with values obtained from typical plant amyloses (iodine affinity 19·2; B.V. 1·20) the calculated proportion of amylose in the C. paramecium starch is 26 - 34%.

Since amyloses from different biological sources have varying properties (e.g. D.P., β - amylolysis limit, iodine affinity) it is necessary to examine in some detail a sample of amylose isolated from the starch under consideration. Because of the marked insolubility of the C. paramecium starch (complete solution could be effected only by shaking in 2N - sodium hydroxide for 24 hours), and the relatively low glucose and high protein and ash contents, it was thought desirable to attempt further purification before fractionation into the amylose and amylopectin components.

A single chloral hydrate extraction (73) carried out at 80°C. in an atmosphere of nitrogen, was unsuccessful. The material recovered on precipitation of the extract with acetone had a glucosan content of only 69% whilst the insoluble residue on similar precipitation yielded a material (63%) containing 85% glucosan. The two fractions were therefore combined and stirred, in an atmosphere of nitrogen, with 0·1N - sodium hydroxide at 100°C. for 10 minutes. The resulting solution, after being separated from an insoluble residue by centrifugation, was neutralised with dilute sulphuric acid and precipitated with acetone to yield 840 mg. of material - "alkali - soluble fraction". This contained 88% glucosan, had an iodine affinity of 6·1 and B.V., 0.65 (λ max., 620 m.μ.). The insoluble residue (440 m.g.) contained 94·5% glucosan and had an iodine affinity of 1·60 and B.V. 0·196 (λ max., 520 m.μ.). A partial separation into components showing marked differences in iodine affinity and B.V. had therefore occurred. This result

is in accord with the report of Baum and Gilbert (74) that centrifugation of dispersions of undamaged starch granules in cold dilute alkali effected fractionation into amylose, which completely dissolved, and amylopectin, which was insoluble.

β - Amylolysis of the alkali insoluble fraction resulted in a 64% conversion into maltose. On oxidation with sodium metaperiodate at 2°C. for 240 hours, 1·15% formic acid was released, corresponding to an average chain length (C.L.) of 25 glucose residues. Allowing for the presence of 14% of amylose of D.P. 330 (see later) the production of formic acid from the amylopectin component is 1·30%; this corresponds to a C.L. of 22. In the course of the oxidation 1·07 moles of periodate were consumed per anhydro-glucose unit showing the absence of 1:3 glucosyl linkages in the starch.

β - Amylolysis of the alkali soluble fraction, using a β - amylase preparation which contained Z - enzyme, resulted in an 81% conversion into maltose. A portion of this amylose - enriched material was fractionated into its amylose and amylopectin components by the method of Robertson and Greenwood (75) which involves precipitation of the amylose with thymol and subsequent purification of the amylose complex by repeated butanol precipitation.

The amylopectin fraction (80 m.g.) contained 82% glucosan. It was considered that the relatively low glucosan content was due to the presence in the amylopectin, which was isolated by freeze-drying the supernatant solution from the thymol precipitation of the amylose, of much inorganic material. Because of the limited amount of material this was not confirmed by determination of the ash content. The iodine affinity, 0·09, and B.V., 0·125 (λ max. ~~475~~ 540 m.μ.) showed that this fraction resembled typical amylopectin and was not contaminated by any appreciable amount of amylose. The absorption spectrum

of the iodine complex, formed under the conditions described in Section IV, was similar to those of typical plant amylopectins (cf. Section IV). Incubation with barley β - amylase resulted in a 60% conversion into maltose. The presence in the molecule of α - 1:6 - glucosidic linkages was shown by the increase in the β - amylolysis limit, after pre-treatment with yeast isoamylase (76), from 60 to 82%. The value of the β - amylolysis limit of C. paramecium amylopectin is similar to that of typical plant amylopectins (β - amylolysis limit, 55 $\pm$ 5%) (77) and, in conjunction with the C.L. value obtained from periodate oxidation studies, shows that the internal chains average 5 - 6 glucose residues, the average external chain length (E.C.L.) being 15 - 16. A comparison of the structural features of C. paramecium amylopectin with amylopectins and glycogens prepared from several plant, animal, and microbiological sources is given in Table 19. It is evident that C. paramecium amylopectin differs markedly in branching properties from the reserve polysaccharides of T. foetus, T. gallinae and T. pyriformis and closely resembles the amylopectin-type polysaccharides synthesised by Cycloposthium, the holotrich ciliates of sheeps rumen, and by waxy maize.

The amylose fraction of the starch (120 mg., 97% glucosan) gave an intense blue stain with iodine. The B.V., 0·98 (λ max., 620 mμ.), was lower than that of typical amyloses [potato amylose B.V., 1·2 - 1·5; λ max., 620 - 650 mμ. (70)] as was the iodine affinity of 10·6 [potato amylose iodine affinity, 19·2 (61)]. These low values could be due either to the presence in the amylose sample of an appreciable amount of contaminating amylopectin or to abnormalities in the molecular structure of the amylose. It has been observed (78) (79) (80) that satisfactory fractionation of certain cereal starches is much more difficult to achieve than the fractionation of potato starch. It was found (80) that two cereal starches prepared from

TABLE 19

Properties of Amylopectin - Glycogen Type Polysaccharides Isolated From Various Sources

Property	Source									
	Trichomonas gallinae	Trichomonas foetus	Tetrahymena pyriformis	Rabbit liver	Dunaliella bioculata	Cyclo-posthium	Holotrich ciliates	Polytomella coeca	Chilomonas paramecium	Waxy maize
$[\alpha]_D$ (water)	+197°	+199°	+195°	+198°	+169°	-	-	-	-	+212°
$[\alpha]_D$ (N - NaOH)	-	-	-	+169°	-	+154°	+171°	+160°	+157°	+153°
Iodine colouration	yellow-brown	yellow-brown	yellow-brown	red-brown	-	purple	red-purple	-	purple	red-purple
B.V.	-	-	-	-	-	0·05	0·10	0·11	0·13	-
β-Amylolysis limit	51	60	44	45	60	-	-	48	60	54
C.L.	9	15	13	13	18	22-24	21-22	-	22	22
E.C.L.	6-7	11-12	8-9	8-9	13-14	-	-	-	15-16	14-15
I.C.L.	1-2	2-3	3-4	3-4	3-4	-	-	-	5-6	6-7

THE RELATIONSHIP BETWEEN THE SPECIFIC VISCOSITY

AND THE CONCENTRATION OF CHILOMONAS PARAMECIUM AMYLOSE

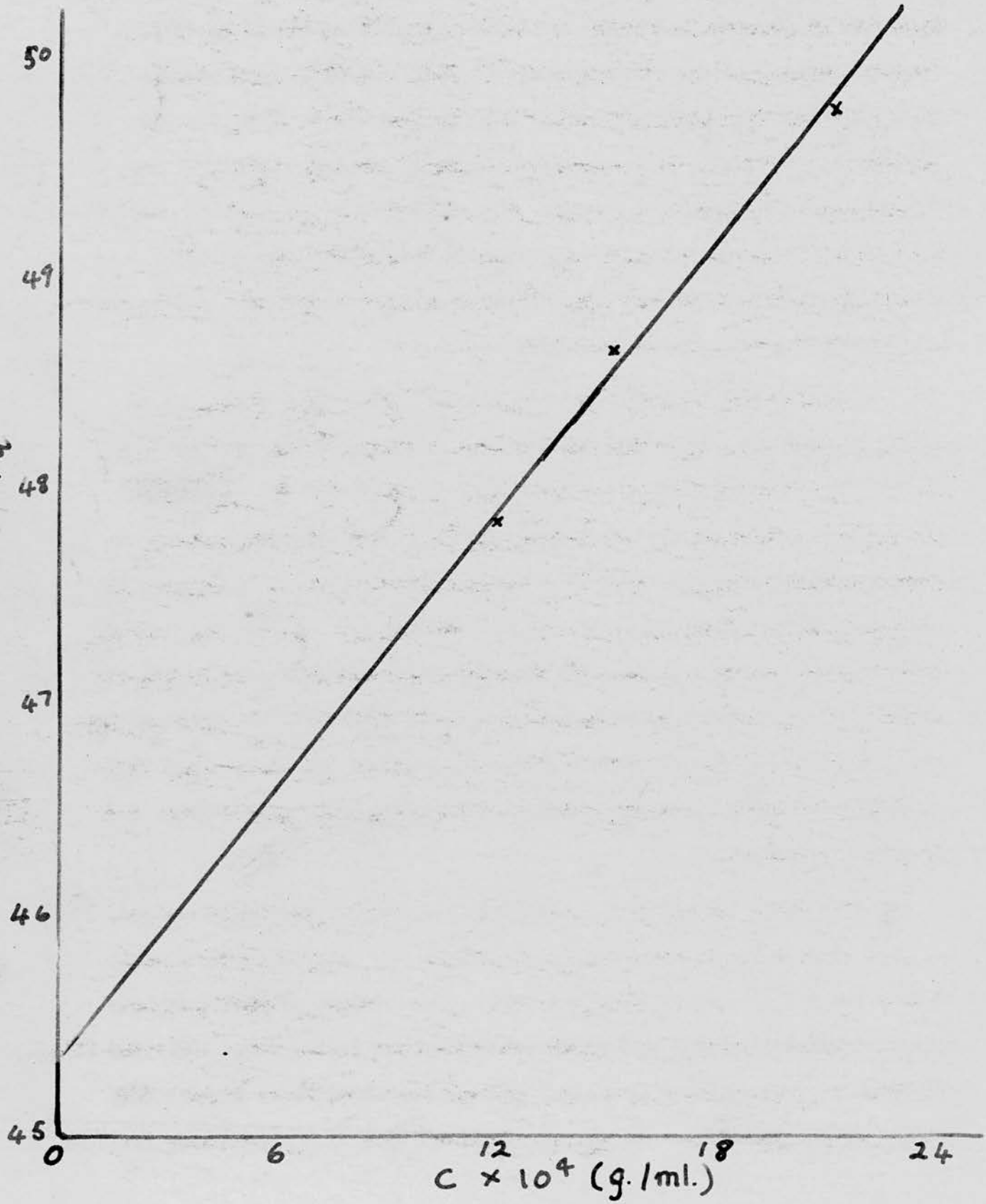


Fig. 13

wheat and oat flour were extremely difficult to disperse prior to conventional fractionation using precipitants. Amylose samples prepared from such dispersions had iodine affinity values of as little as 13·7. Aqueous leaching of the starch granule at 70°C. and 98°C. gave samples having the highest iodine affinities (oat amylose, 19·1, 18·0, : wheat amylose 17·8, 15·8). It was observed, however, that one sample of oat amylose, which had an initial iodine affinity of 11·0, on repeated precipitation with butanol gave a material having an iodine affinity of 16·8. It is suggested that the low iodine affinities are due to the presence in the amylose of large amounts of amylopectin.

On digestion with barley β - amylase (containing Z enzyme), C. paramecium amylose was hydrolysed to maltose (95%). With purified soya bean β - amylase (which did not contain Z enzyme) the β - amylolysis limit was 90%, showing that the number of Z - labile linkages present was very much smaller than in typical potato amyloses which are hydrolysed to only ca. 70% when digested with pure β - amylase (77) (82). The failure of the barley enzyme to bring about complete degradation may be due to the presence in the amylose of a maximum of ca. 12% amylopectin impurity. This can not account fully for the low iodine affinity of the amylose and it is probable that the C. paramecium amylose differs in some respect (e.g. D.P.) from potato amylose.

The degree of polymerisation (D.P.) of the C. paramecium amylose, calculated from the limiting viscosity number, $[\eta]$, by the relationship D.P. = 7·4 x $[\eta]$ ..... (81), was 330. D.P. values of potato amylose prepared under carefully controlled conditions are in the range 1000-5000 (81). Although several amyloses from other sources have been found to have D.P. values of ca. 500 (77). It has been observed (82) that the amylose fraction

of starch tends to be degraded by chloral hydrate. It seems probable that the low D.P. is not a property of the native C. paramecium amylose but is the result of inadvertent degradation during the extraction.

The amylose content of the original starch and of the various fractions may be calculated from iodine affinity values of each fraction and of the pure amylose. Similar, though less accurate, estimations can be made by comparison of B.V. or β - amylolysis limit of the various fractions and of the pure amylose and amylopectin. Such calculations may be made on the basis that

(a) the amylose fraction was pure (iodine affinity, 10·6; B.V. 0·98; β - amylolysis limit 95),

or (b) the amylose was contaminated by 12% amylopectin (for pure amylose, therefore, iodine affinity is 12·0; B.V., 1·10, β - amylolysis limit, 100).

Results thus obtained are listed in Table 20.

TABLE 20

Sample	% Amylose calculated from					
	(1) Iodine Affinity		(2) B.V.		(3) β - limit	
	(a)	(b)	(a)	(b)	(a)	(b)
Whole starch	47	42	48	42	43	38
Alkali insoluble fraction	15	13	8	7	11	10
Alkali soluble fraction	58	51	62	54	60	53

The values obtained from potentiometric iodine titration are the most accurate. The amount of amylose in the original starch is therefore 45 $\pm$ 5%. This is a much higher proportion of amylose than is present in

most plant starches (ca. 25% (83)), although starches from beans, peas, and related seeds contain 30 - 68% amylose (83).

Of the three protozoal starch-type polysaccharides which have been previously investigated, that from the flagellate Polytomella coeca contained 13 - 16% amylose whereas in those from Cycloposthium and the ciliates of sheep's rumen no amylose could be detected. There is, however, a close structural resemblance among the amylopectins synthesised by these latter protozoa and by waxy maize. It has been shown that the degree of branching in synthetic (in vitro) amylopectins is dependant on the relative amounts of phosphorylose and Q- enzyme present in the digest (84). In view of the similar branching properties of waxy maize starch and C. paramecium amylopectin it seems probable that the ratio of phosphorylase to Q enzyme in waxy maize is similar to that in C. paramecium. This being so it is of very great interest that a large amount of amylose (45%) is present in C. paramecium starch while waxy maize is virtually free of amylose. The in vitro syntheses of amylose and of amylopectin throw no light on the problem of how these two components are synthesised in the same starch granule. The suggestion (70) that, in the granule, the enzyme molecules become surrounded by their products of synthesis which then prevent the free movement of the enzymes, can account for the presence of amylose and amylopectin in the same granule. In this case it might be expected that a high proportion of Q- enzyme would give rise not only to a highly branched amylopectin but also to a low proportion of amylose. In fact, however, there does not appear to be any correlation between the branching properties of the amylopectin and the amount of amylose present. An alternative mechanism has been proposed by Whelan (70) who suggests that the starch synthesising system may contain two compartments separated by a semi-permeable

membrane. In one compartment amylopectin is synthesised from maltotetraose or maltopentaose by the successive actions of D- and Q- enzymes. The glucose which results from the disproportionation of the oligosaccharides then diffuses through the membrane and is converted into glucose - 1 - phosphate which, in the presence of a primer, e.g. maltotetraose, then undergoes phosphorylation to give amylose. On this hypothesis, waxy maize starch granules should possess no membrane system. Much work remains to be done, however, before any explanation can with confidence be proposed.

Conclusions.

1. A sample of starch from Chilomonas paramecium has been examined and found to be generally similar to typical plant starches.

2. The amount of amylose present (45%) is greater than in most plant starches.

3. The amylose component has been isolated and found to differ in iodine affinity and B.V. from typical amyloses. It is suggested that these differences are due, in part, to the low D.P. (330) of the sample. It is probable that this low D.P. is not a property of the natural amylose but was caused by inadvertent degradation during the extraction.

4. The amylopectin component has been found to be similar in properties to typical plant amylopectin.

III. EXPERIMENTAL.

The sample of starch examined was prepared by Dr. J. F. Ryley. Pure cultures of C. paramecium were grown in the dark at 25 - 30°C. in 2 l. flasks containing 1·5 l. of aqueous medium composed of 0·1% sodium acetate and 0·1% Oxoid brand "Lab - Lemco" beef extract at pH 6 - 6·5. The starch was isolated from the cells by extraction with chloral hydrate (73) followed by acetone precipitation. Contaminating chloral hydrate was removed by extraction of the starch, in a soxhlet apparatus, with methanol followed by ether.

Examination of the Whole Starch

1. Complete acid hydrolysis.

Chromatographic examination of the complete acid hydrolysate (solvent (1); spray (1)) showed glucose to be the only reducing sugar present.

2. Reaction with acid - resorcinol reagent (72).

The reagent consists of copper sulphate decahydrate (45 mg.), water (50 ml.), glycerol (A.R., 130 g.) and concentrated hydrochloric acid (100 ml.). Carbohydrate (10 mg.) was heated with the reagent (5 ml.) and resorcinol (0·45% w/v in water; 1 ml.) for 12 minutes on a boiling water bath. The solution was then cooled and the light absorption measured using a Spekker absorptiometer at 520 m.μ. A graph of m.g. carbohydrate against light absorption gave straight lines of the following slopes.

Fructose	3·0	scale	divisions	/	m.g.	fructose
Maltose	0·016	"	"	"	/	m.g.	glucose
Soluble Starch	0·014	"	"	"	/	m.g.	glucose
C. paramecium starch	0·011	"	"	"	/	"	"

It is concluded that fructose is not a constituent of C. paramecium starch since the reaction is less than that of maltose.

3. Glucose content.

The starch (9·44 m.g.) when hydrolysed by the method of Pirt and Whelan (85) gave glucose (8·15 m.g.), equivalent to 78·4% glucosan.

4. Ash content.

The starch (18·73 m.g.) gave ash (1·81 m.g.) equivalent to 9·7%.

5. Protein nitrogen.

On analysis (method 6) the starch (47·9 m.g.) gave a Spekker absorptiometer reading of 0·090 equivalent to 0·45 m.g. nitrogen or 6% protein.

6. Specific rotation.

The starch (53·8 m.g.) was moistened with alcohol and shaken overnight with 2 N- sodium hydroxide (5 ml.) in a standard flask (10 ml.). The solution was diluted to 10 mls. with water and the optical rotation measured and found to be 0·66° equivalent to $[\alpha]_D$ + 157°.

7. Digestion with β - amylase.

The preparation of β -amylase used was a commercial preparation purchased from the Wallerstein Laboratories (New York); it was free from

maltase and α - amylase but contained Z enzyme since it could hydrolyse a true amylose β - limit dextrin. The enzyme powder had an activity of 108 units per m.g. determined by Hobson, Whelan and Peats' method (86).

The starch (12·29 m.g.) was dissolved in 2 N - sodium hydroxide solution (5 ml.) in a standard flask (25 ml.). The solution was neutralised (phenolphthalein indicator) with sulphuric acid. 0·2 M - acetate buffer (5 ml., pH 4·6) and β - amylase solution (6 m.g., 648 units; 2 ml.) were added, the solution made to 25 ml. with water and incubated at 37°C. Aliquots (5 ml.) were removed at intervals and the reducing power determined using the Somogyi reagent. The reducing power (which was constant at 24 hours incubation) was equivalent to the production of 7·60 m.g. maltose. The β - amylolysis limit was therefore 75%.

8. Determination of B.V. (162)

The dry sample (10 - 15 m.g.) contained in a standard flask (10 ml.) was dissolved in 2 N - sodium hydroxide (2 ml.) and the solution diluted to 10 ml. with water. Aliquots (1 ml.) were removed and the starch concentration determined by quantitative acid hydrolysis. A volume (4 - 5 ml.) of solution containing 5 m.g. starch was introduced into a standard flask (500 ml.) the volume being made up to 100 ml. with water. 3 N - Hydrochloric acid (0·5 ml.) was added followed by a solution of 0·2% iodine in 2% potassium iodide (5 ml.). The solution was diluted to 500 ml. and the light absorption measured, against a reagent blank, in a Spekker absorptiometer using 4 cm. cells and red '600' filters. The wavelength of maximum light absorption was found by examination of this solution using a Unicam S.P. 600 spectrophotometer (1 cm. cells).

Results:- B.V., 0·54.

λ max., 590 m.μ.

9. Potentiometric iodine titration.

The iodine binding power of this and subsequent samples was quantitatively determined by Dr. A. W. Arbuckle, using the potentiometric iodine titration method described by Anderson and Greenwood (61).

Results are shown graphically (Fig. 12).

Extraction with Chloral Hydrate (73).

The starch (1·6 g.) was placed in a three-necked flask (250 ml.) and maintained with stirring at 80°C. for 1 hour in 33% aqueous chloral hydrate solution (100 ml.) under an atmosphere of nitrogen. The gelatinous residue was collected by centrifugation and extracted with two further portions (50 ml.) of chloral hydrate solution. The combined extracts were filtered through a sintered glass crucible (porosity 3) and injected in a fine stream into acetone (2 vols.). The resulting flocculent precipitate was collected and dried with acetone and ether. The last traces of chloral hydrate were removed by extraction in a Soxhlet apparatus with acetone and then ether. Yield of ether dry material, 560 m.g. Glucosan content 69%. On similar precipitation the gelatinous residue yielded 969 m.g. material which contained 85% glucosan.

Extraction with Sodium Hydroxide.

The combined fractions from the chloral hydrate extraction (1·5 g.) were stirred for 10 minutes at 100°C. with 0·1 N - sodium hydroxide in an atmosphere of nitrogen. After cooling and neutralisation (dilute sulphuric acid, phenolphthalein indicator) an insoluble gelatinous residue was removed by centrifugation. Acetone precipitation of the solution yielded 840 m.g. material - "alkali soluble fraction". Precipitation of the insoluble

residue gave 440 m.g. of "alkali insoluble fraction".

Properties of the Alkali Insoluble Fraction

Found:-

94·5% glucosan
2·7% ash
B.V., 0·196; λ max., 520 m.μ.
Iodine Affinity, 1·60.

Periodate Oxidation.

Alkali insoluble starch (160 m.g.) was moistened with alcohol and shaken overnight with 2N - sodium hydroxide (10 ml.) in a standard flask (25 ml.). The solution was neutralised with sulphuric acid (methyl red indicator) and 20 mls. of solution (equivalent to 121·2 m.g. glucosan) pipetted into a standard flask (25 ml.). Sodium metaperiodate solution (3 ml., 8%) was added together with water to 25 ml. A periodate blank was also prepared. Both reaction mixtures were placed in the dark at 2°C.; aliquot portions were removed at intervals and the release of formic acid and uptake of periodate determined.

Results: Sodium hydroxide solution, 0·0102 N.
Iodine solution, 0·0905 N.

(a) Formic acid release.

Samples (3 ml.) were taken and excess periodate neutralised by the addition of ethylene glycol (0·5 ml.).

TABLE 20

Time (days)	Digest Titre (ml.)	Blank Titre	Corrected Titre	Formic Acid * production (%)
0	0·04	0·00	0·00	-
7	0·34	0·00	0·30	0·966
10	0·40	0·00	0·36	1·16
12	0·39	0·00	0·35	1·13

* expressed as m.g. formic acid/100 m.g. polysaccharide.

(b) Periodate uptake.

Samples (2 ml.) were removed and added to arsenite (5 ml.) and sodium bicarbonate (1·5 g.). Excess arsenite was determined by back titration with iodine.

TABLE 21

Time (days)	Digest Titre (ml.)	Blank Titre (ml.)	Corrected Titre (ml.)	Periodate * Uptake
7	3·22	4·60	1·38	1·04
10	3·18	4·60	1·42	1·07
12	3·18	4·60	1·42	1·07

* expressed as moles periodate consumed per anhydro glucose unit.

β - Amylolysis.

Alkali insoluble starch (15·4 m.g. glucosan) was incubated with barley β - amylase as before. The β - amylolysis limit was 64%.

Properties of the Alkali Soluble Fraction.

Found:-

glucosan content 88%
ash content 5·9%
B.V., 0·65 (λ max., 620 m.μ.).
β - amylolysis limit, 81%
Iodine affinity, 6·1.

Fractionation of the Alkali Soluble Material.

Starch (500 m.g.) was suspended in water (20 ml.) and added to vigorously stirred boiling water (65 ml.) in an atmosphere of nitrogen. The mixture was boiled for 20 minutes and then allowed to cool. The insoluble residue was removed by centrifugation. The solution was heated to 60°C., powdered thymol (1·5 g.) was added and the mixture stirred at 60°C. for 30 minutes and then kept at room temperature for 3 days. The thymol complex was removed by centrifugation and the supernatant solution freeze-dried to give amylopectin. This was heated under reflux with methanol in a Soxhlet apparatus to remove thymol, redissolved and freeze-dried. The thymol complex was directly dispersed in boiling water (50 ml., under nitrogen), re-distilled butanol (5 ml.) added and the mixture stirred at 95°C. for 30 minutes and allowed to cool slowly to room temperature. The butanol complex was removed by centrifugation. This was purified by two further precipitations with butanol and the amylose 'crystallised' by stirring with butanol.

Yield:- amylose, 129 m.g., amylopectin, 80 m.g.

Properties of the Amylopectin Fraction.

Found:-

glucosan content, 82%
B.V., 0·125 (λ max., ~~475~~ 540 m.μ.)
β - amylolysis limit, 60%
Iodine affinity, 0·09.

Action of iso amylase.

Yeast iso amylase was prepared by Miss Zeenat Gunja using the method described by Manners and Khin Maung (76) (87).

Amylopectin (9·78 m.g.) was dissolved in 0·2N - sodium hydroxide (2 ml.), neutralised, 0·2 M- acetate buffer (5 ml., pH 5·8) was added followed by iso amylase (10 m.g., 2 ml.). The digest was maintained at room temperature for 18 hours and the enzyme then inactivated by heating for 3 minutes at 100°C. After cooling, barley β - amylase (5 m.g., 1 ml.) was added, the volume made to 25 ml., and incubated at 35°C. for 36 hours. Before sampling, precipitated protein was removed by centrifugation. 6.98 m.g. maltose was produced equivalent to a β - amylolysis limit of 82%.

Properties of the Amylose Fraction

Found:- Glucose content, 97%
B.V., 0·98 (λ max., 645 m.μ.)
Iodine affinity, 10·6.

β - amylolysis

(a) Barley β - amylase.

Amylose (10·35 m.g.) was incubated with barley β - amylase as before. The β - amylolysis limit was 95%.

(b) Soya-bean β - amylase.

Pure soya bean β - amylase was prepared by Dr. I. D. Fleming using the method of Peat, Pirt and Whelan (88); it was free from α - amylase and Z enzyme and had only negligible maltase activity.

Amylose (18·45 m.g.) was dissolved in 0·2M - acetate buffer (5 ml., pH. 4·6) and soya bean β - amylase (0·1 ml., 1000 units) added, the solution

THE MODIFIED UBBELOHDE VISCOMETER

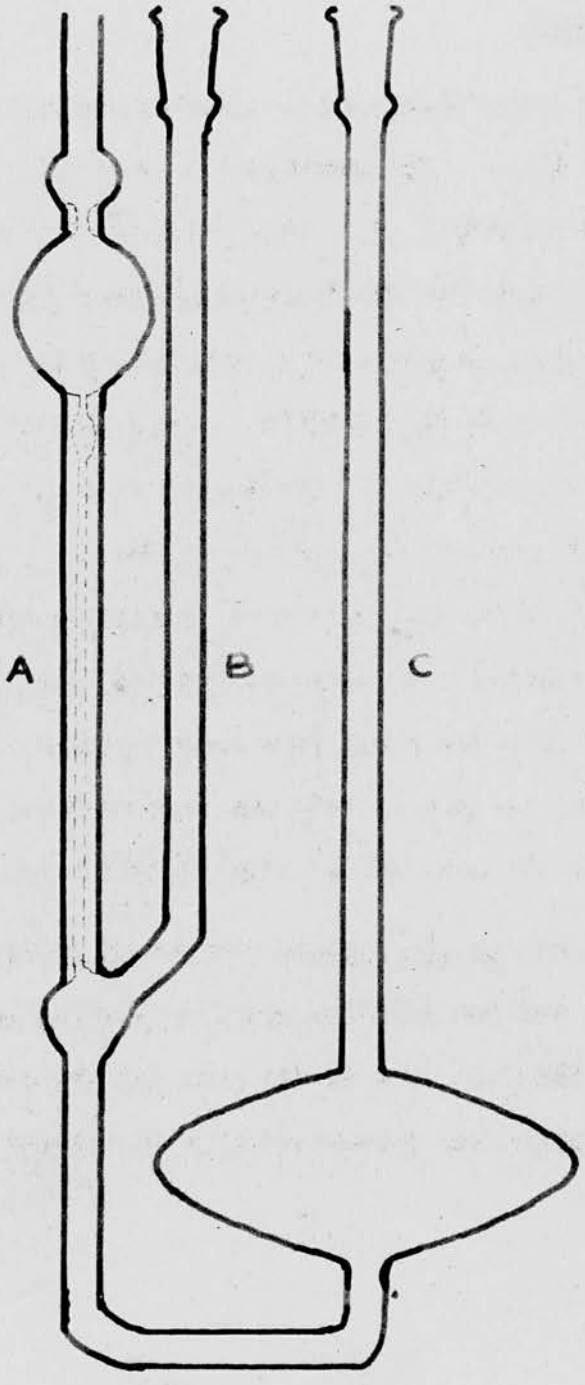


Fig. 14

made to 25 ml. and incubated at 35°C. for 24 hours. 17·0 m.g. Maltose was produced equivalent to a β - amylolysis limit of 90%.

Viscometric Determination.

Amylose (35 m.g.) was dissolved by shaking overnight in 0·2M - potassium hydroxide (15 ml.). The solution was then filtered through a sintered glass crucible (porosity 4). This solution (13 ml.) was added by pipette down tube A of a modified Ubbelhode viscometer (Fig. 14) which was clamped firmly in a vertical position on a brass stand and placed in a bath thermostatically controlled at 25 ± 0·001°C. After allowing the solution to come to temperature equilibrium (20 minutes) tube B was closed with a ground glass stopper and pressure was applied at the top of A, thus transfering a quantity of liquid to the bulbs above capillary tube C (0·4 m.m.) The pressure was then released, the stoppers removed, and the time required for the liquid level to pass two marks (one above and one below D) was measured. A stop watch, reading to 0·02 sec. was used and the flow time of the solution taken as the mean of two such observations.

Dilutions were made *in situ*, given volumes of solvent being added by pipette down tube A, and the solution mixed by blowing gently down tube B a number of times. The flow time of the pure solvent was also determined. The concentration of amylose was determined by acid hydrolysis of the filtered solution.

TABLE 22

C (g./ml.)	T	To	T - To	$\frac{T - To}{To} \times \frac{1}{C}$
0·00218	716·56	646·37	70·19	49·81
0·00157	695·78	646·37	49·41	48·68
0·00123	684·45	646·37	38·08	47·89

C is concentration of amylose (g./ml.)
T is flow time of solution (secs.)
To is flow time of solvent (secs.)

The specific viscosity, $\eta_{sp.}$, is given by $\frac{T - To}{To}$

$\frac{\eta_{sp.}}{C}$ is known as the Viscosity Number and by extrapolation of the graph, $\eta_{sp.}/_{C}$ against C, to zero concentration, the limiting viscosity number $[\eta]$ is obtained. This latter is characteristic of the polymer solution and from it the molecular weight of the polymer can be obtained.

It has been shown (81) that for amylose, D.P. = $[\eta] \times 7{\cdot}4$.

From the graph (Fig. 13),

$$[\eta] = 45{\cdot}4$$

and hence D.P. = 335

SECTION VI

A PRELIMINARY EXAMINATION OF THE POLYSACCHARIDES SYNTHESISED BY OCHROMONAS MALHAMENSIS.

I. INTRODUCTION.

Ochromonas malhamensis is a free-living flagellate which is found in fresh and brackish water. It is a chrysomonad and is related to the brown algae. It possesses a yellow photosynthetic pigment and is a facultative phagotroph, capable of growth and reproduction in the dark (89). A considerable volume of work has recently been done on the metabolism of this organism which possesses highly specific requirements for vitamin B_{12}. In view of this, cultures of Ochromonas malhamensis have been used in the biological assay of vitamin B_{12} (90) (100).

In the present work an examination has been made of polysaccharide material isolated from the cells by aqueous extraction.

II. DISCUSSION.

The materials obtained from Dr. Ryley were prepared by ethanolic precipitation of hot-water extracts of cells of Ochromonas malhamensis which had been grown in daylight. Two such samples were examined and a report is given below of the purification and structural investigation of these samples. The results obtained from periodate oxidation studies are expressed, unless otherwise stated, in moles of formaldehyde or formic acid produced, or of periodate consumed, per monosaccharide residue

Sample I

This material was stirred with cold water, centrifuged, and ethanol added to the supernatant solution. The resulting precipitate was purified by repeated solution in water, centrifugation and precipitation with ethanol to yield a buff coloured powder which was readily soluble in water, giving a yellow-brown opalescent solution. Chromatographic examination of an acid hydrolysate showed the presence of glucose, smaller amounts of galactose and mannose, and a trace of xylose. The powder contained 79% polysaccharide (calculated as glucosan from the reducing power of the acid hydrolysate), 3·4% ash and 13·3% protein. A partial acid hydrolysate contained, in addition to monosaccharides, sugars having the same chromatographic mobility as laminaribiose and laminaritriose. The presence of a proportion of 1:3 - glycosidic linkages was also indicated by the consumption, on complete oxidation, of only 0·34 moles of periodate.

An attempt to remove the contaminating protein by shaking a solution

of the material with toluene was only partially successful; the material recovered after this procedure had been repeated twelve times still contained 5·7% protein.

Successful separations of polysaccharides by precipitation with Cetavlon (cetyl trimethyl ammonium bromide) have been reported (101) and a solution of the polysaccharide material was therefore treated with Cetavlon in an attempt to separate the polysaccharide components. A brown precipitate, an acid hydrolysate of which contained traces of galactose, glucose and mannose in approximately equal amounts, was removed by centrifugation. Polysaccharide material was then isolated from the supernatant solution by the addition of ethanol. The resultant white powder, which contained 90% polysaccharide (calculated as before), 1·9% ash and 4·8% protein, was readily soluble in water giving a clear colourless solution. Paper chromatography of an acid hydrolysate showed that the same monosaccharides were present as before. Visual comparison indicated that some of the galactose-containing material had been preferentially precipitated by the Cetavlon.

On partial acid hydrolysis, oligosaccharides having the same chromatographic mobility as standards of laminaribiose, laminaritriose and laminaritetraose were produced together with higher, related oligosaccharides. No other oligosaccharides could be detected by paper chromatography. The same series of oligosaccharides were formed on digestion of the polysaccharide with an endo- β - glucosidase preparation prepared from Cladophora rupestis (102) by Dr. W. A. M. Duncan. Apart from the monosaccharides present, the chromatograms obtained from the above hydrolysates were identical to those obtained on similar treatment of laminarin. This indicates the presence of a large proportion of β - 1:3 - glucosidic linkages in O. malhamensis polysaccharide. On incubation of O. malhamensis polysaccharide with almond

emulsin (an exo- β - glucosidase) prepared by Dr. F. B. Anderson (103), faint traces of glucose only could be detected. Laminarin was also only slightly hydrolysed under these conditions.

Since visual estimation indicated that the glucose present in the acid hydrolysate represented only ca. 75% of the total reducing sugars present, various methods of purification were attempted:-

(1) It was found that the material did not form a copper complex when mixed with Fehlings solution.

(2) An aqueous solution of the polysaccharide at pH. 5·0 was mixed with Cetavlon solution. On raising the pH. to 9·0 a precipitate formed. This was removed by centrifugation and the material remaining in solution was recovered by precipitation with ethanol. Chromatographic examination of the acid hydrolysates showed that the proportion of monosaccharides present was approximately the same in each fraction.

(3) Acetone was slowly added to an aqueous solution of the polysaccharide and the resulting precipitates were removed by centrifugation, hydrolysed and examined chromatographically. The first fraction contained galactose, glucose and mamose in the ratio 2:1:1, the second and third fractions contained little or no galactose, a trace of xylose, and glucose and mannose in the ratio 5:1.

From the above results it is apparent that fractionation into different polysaccharide components has been achieved only by the use of acetone.

In order to follow such fractionations quantitatively, it was necessary to find a method of determination of the individual sugars present. It was considered that methods involving the paper chromatographic separation of

the monosaccharides prior to quantitative analysis would be inaccurate since the separation of glucose and mannose is incomplete, for example, the Rg. value of mannose in solvent 1 is 1·15. A method involving the quantitative and selective destruction of glucose present in a hydrolysate was therefore employed. This was effected by the use of a commercial preparation of the highly specific glucose oxidase (104). In control experiments using solutions containing glucose (80%) and mannose (20%), it was found that the destruction of glucose, under the conditions employed, was complete in ca. 30 hours. The amounts of glucose and mannose present were then calculated from the reducing powers (Somogyi reagent) at zero time and on complete oxidation (30 - 48 hours). Values thus obtained for the amount of glucose present were 102 - 104% of theoretical, that for mannose being 96% theoretical. These results are satisfactory and this method has therefore been employed for the estimation of the glucose and mannose present in acid hydrolysates of the various polysaccharide fractions. In the results quoted below, no correction is made for the slight oxidation of mannose since this is of the same order as experimental error.

The remainder of the purified Sample 1 material (2·0 g.) was dissolved in water and fractionally precipitated with acetone. The first fraction (445 m.g.) contained galactose and slightly smaller amounts of glucose and mannose; the second (771 m.g.) contained glucose, a little mannose, and a trace of galactose. Fraction 3, which contained glucose, a little mannose and a trace of xylose, was dissolved in water and precipitated with acetone to yield two fractions, the first of which (fraction 3A) was free from xylose. On analysis as above, fraction 2 was found to contain 71·0% anhydro-glucose and 11·1% anhydro-mannose; sample 3A contained 77·9% anhydro-glucose and 11·3% anhydro-mannose. Since the proportion of glucose to mannose was

approximately the same, these two fractions were combined and dialysed against distilled water to yield 770 m.g. of material (fraction 4).

This fraction contained 84·7% anhydro-glucose and 12·2% anhydro-mannose, the total reducing sugar content thus being 96·9%. The R.P. (relative to laminaribiose) was 63, $[\alpha]_D$ + 10·8 (C, 1·0 in water).

A portion of fraction 4 was oxidised with sodium metaperiodate at 2°C. (cf. Section III). The production of formic acid after six days oxidation was 0·078 moles. The oxidation mixture was then brought to room temperature and stored in the dark for a further two days. The formic acid release at this time was 0·081 moles showing that the primary oxidation was complete. The periodate uptake after six and eight days was 0·17 and 0·18 moles respectively. In a duplicate experiment at 2°C., the formic acid release after seven and nine days oxidation was 0·080 and 0·085 moles respectively, the periodate uptake being 0·16 and 0·18 moles. The formaldehyde release was also measured; this was constant after seven to nine days and amounted to 0·029 moles. A portion of fraction 4 was then oxidised with excess periodate at 35°C. in 0·05 M - phosphate buffer pH. 8·0 (105). The formaldehyde production was constant after 18 hours oxidation and amounted to 0·53 moles.

Sample 2

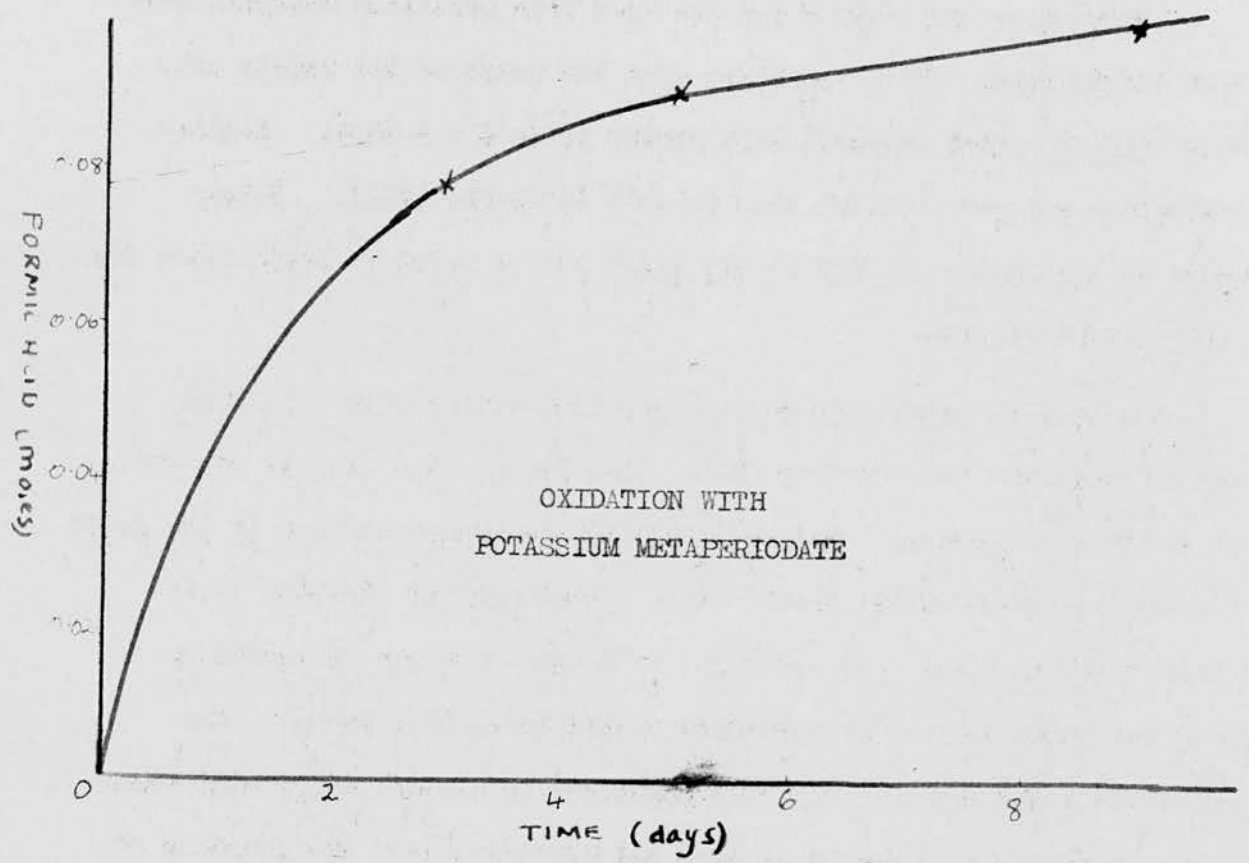
A second sample (8·0 g.) was obtained from Dr. Ryley, and was purified by aqueous extraction and Cetavlon precipitation as before to yield a white powder (5·6 g.). Fractional acetone precipitation of this gave three fractions. Fraction A (1·91 g.) contained anhydro-glucose (56·9%), anhydro-galactose and anhydro-mannose (ca. 11·7%). Fraction B (2·90 g.) contained anhydro-glucose (73·5%), anhydro-mannose (8·6%) and a very faint trace of

galactose. Fraction C (0·31 g.) contained anhydro-glucose (62·1%), anhydro-mannose (11·3%) and a trace of xylose. The ratio of glucose to mannose in fraction B (100:12) was approximately the same as that in fraction 4 of sample 1 (100:14) and it was considered that no greater separation could be effected by acetone fractionation.

A portion of fraction B (500 m.g.) was dispersed in formamide; pyridine and acetic anhydride were added. The resulting acetate was recovered by pouring the mixture into ice-water, dissolved in chloroform and precipitated with petroleum ether (106). The resultant white powder (480 m.g.) had an acetyl content (107) of 41%, $[\alpha]_D$ - 59·3° in chloroform. Attempts to fractionate the polysaccharide acetate were not successful. After fractionation of a chloroform solution of the acetate with petroleum ether, deacetylation (barium methoxide) gave two polysaccharide fractions which on further purification were found to contain:- fraction 1, 82·1% anhydro-glucose, 9·5% anhydro-mannose; fraction 2, 80·9% anhydro-glucose, 9·7% anhydro-mannose.

Fraction B (2·0 g.) was then dissolved in water and fractionally precipitated with acetone. The first fraction "B1" (0·23 g.) contained anhydro-glucose (73·6%) and anhydro-mannose (8·5%); a small amount of galactose was also present. The second fraction contained anhydro-glucose (78·2%) and anhydro-mannose (8·8%) only. This latter fraction was dialysed against distilled water, filtered through asbestos, and precipitated with ethanol to yield a white powder "fraction D" (1·05 g.) containing 85·1% anhydro-glucose and 10·2% anhydro mannose. This had $[\alpha]_D$ + 9·1° in water, and R.P., 63 (laminaribiose standard). On chromatographic examination of a partial acid hydrolysate only oligosaccharides belonging to the laminaribiose series could be detected.

PERIODATE OXIDATION OF FRACTION D.



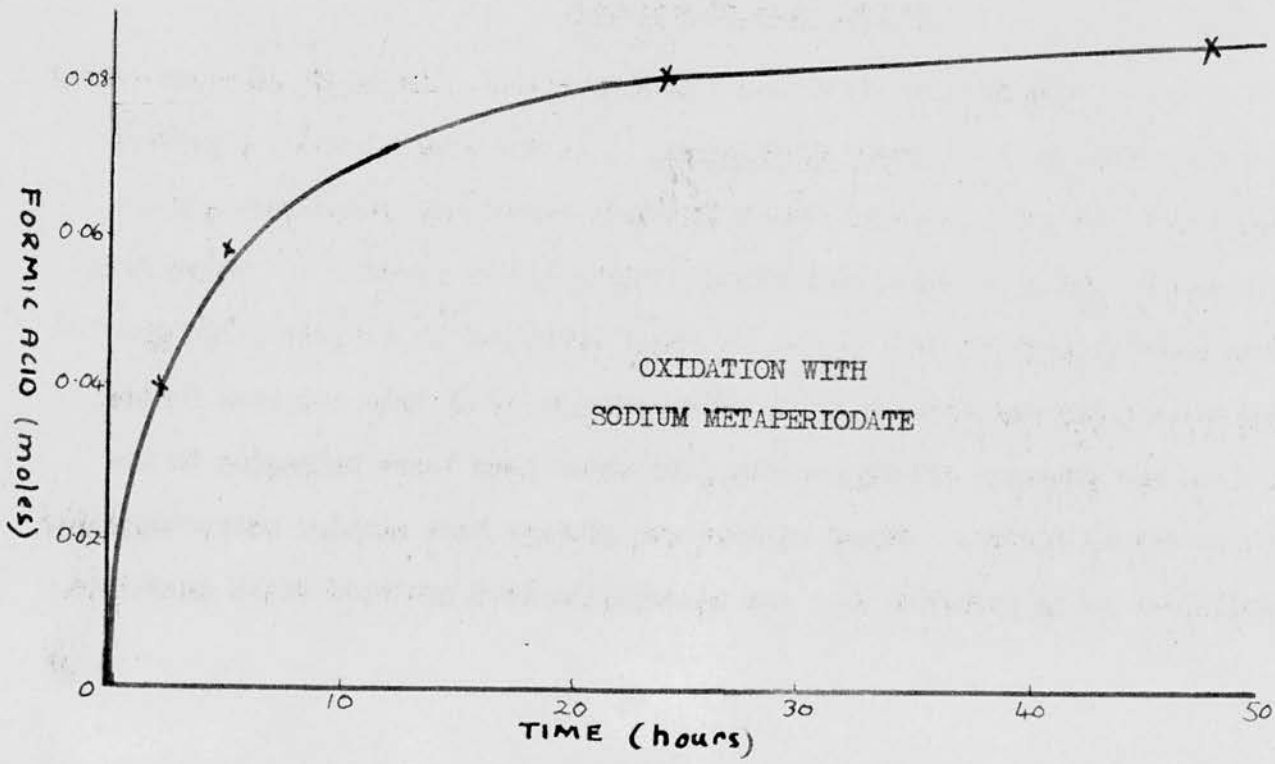


Fig 15

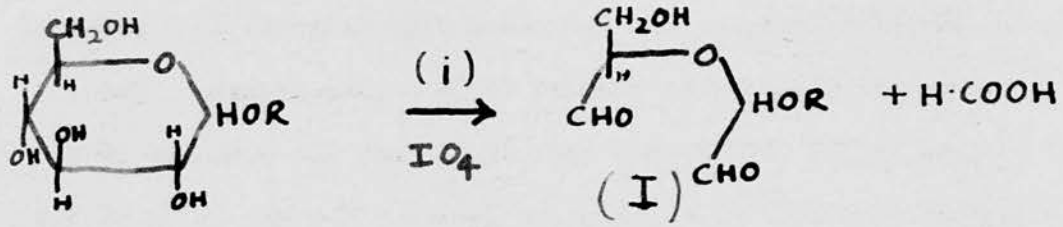
A portion of fraction D was oxidised with potassium metaperiodate at room temperature. It is apparent from the graph of the formic acid release (Fig. 15) that overoxidation occurs after 4 - 5 days. Similar overoxidation has been noticed with soluble laminarin (103). Extrapolation of the linear portion of the graph gave a value of 0·078 moles for the formic acid release.

A sample was then oxidised at room temperature with a limited excess of periodate (cf. Section III). The formic acid release was 0·082 after 24 hours oxidation. Extrapolation of the linear portion of the graph gave a value of 0·078 moles formic acid. Laminarin is likewise fully oxidised after 24 hours (103) (108). After 24 hours the formaldehyde release was 0·025 moles, the periodate uptake being 0·15 moles. The periodate-oxidised material was then recovered by precipitation with ethanol. Chromatographic examination of an acid hydrolysate showed the presence of mannose and glucose. On overoxidation of fraction D, 0·52 moles of formaldehyde were produced.

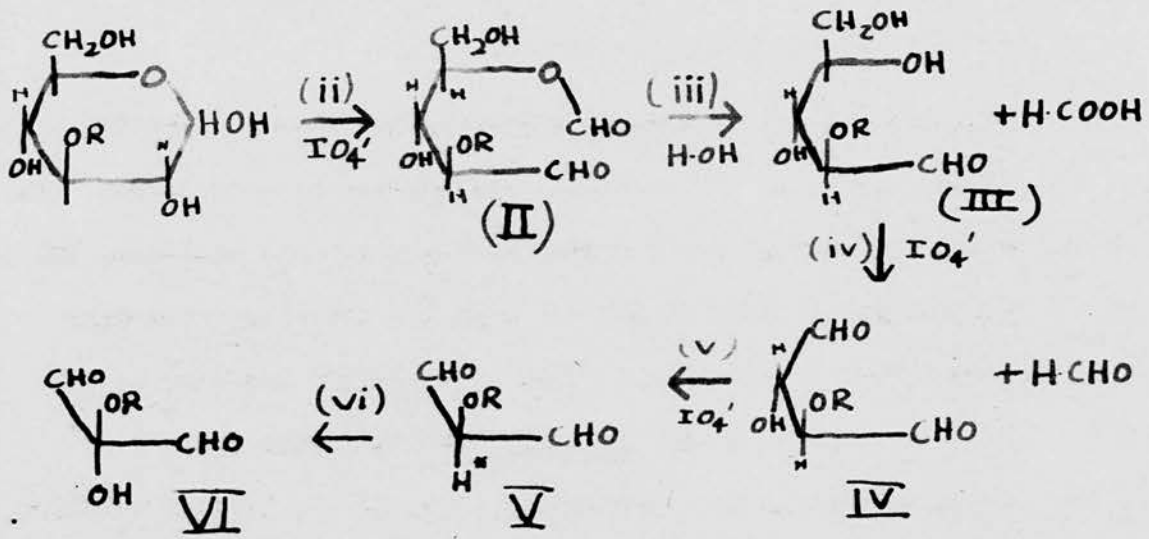
Correlation of Results.

The above results show that a heterogeneous mixture of polysaccharides is synthesised by Ochromonas malhamensis. In the present work, a partial separation has been effected into a fraction containing galactose, glucose and mannose and a fraction containing glucose and mannose. The proportion of mannose present in this latter fraction could not be reduced below ca. 11%. Chromatographic examination of an acid hydrolysate of this material failed to show the presence of oligosaccharides other than those belonging to the laminaribiose series. Since mannose and glucose have similar chromatographic mobilities it is probable that any oligosaccharides produced which contained

PERIODATE OXIDATION OF A 1:3 - LINKED ALDOHEXOPYRANOSAN

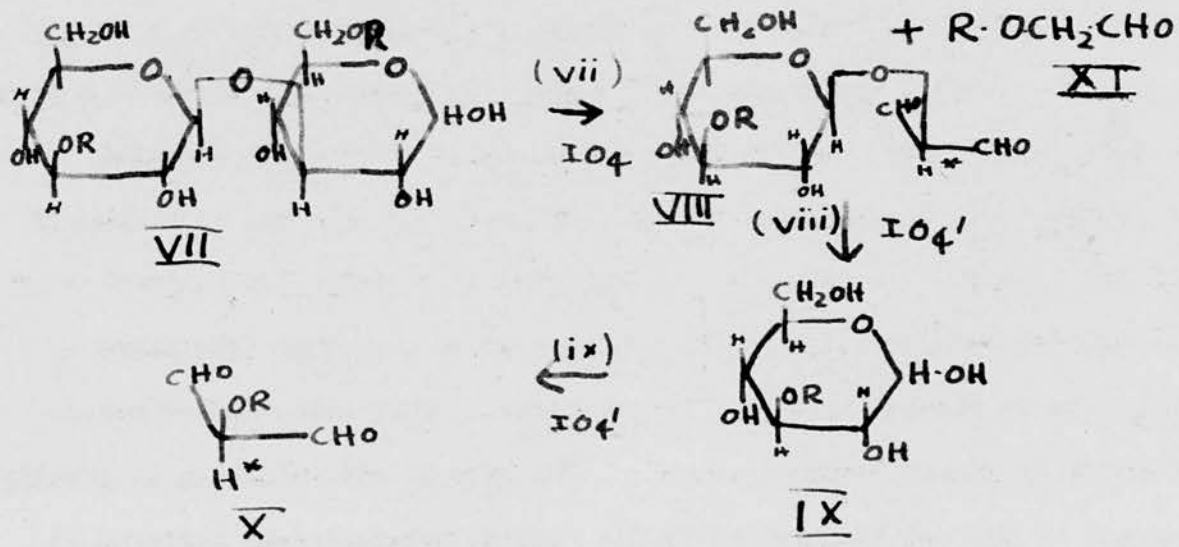


16 a.



H" activated hydrogen atom

16 b.



16 c

Fig. 16.

mannose would have the same chromatographic mobility as the laminarisaccharides. It is not known therefore whether the polysaccharide material is a physical mixture of a mannan and a glucan or whether it is a glucomannan. The products of partial acidic and enzymic hydrolysis show the presence of a large proportion of β - 1:3 - glucosidic linkages. The low value of the specific rotation of the polysaccharide and of its acetate are also indicative of β - glycosidic linkages. That these linkages are 1:3 is also shown by the low value of the periodate uptake.

Before considering the results of periodate oxidation, a short consideration of the action of 1:3 - linked aldohexosans is here given. In a molecule composed entirely of 1:3 - linked aldohexopyranose residues, the non-terminal residues do not possess the α - glycol groupings necessary for Malapradian oxidation. Normal oxidation is therefore confined to the terminal residues. Oxidation of the non-reducing end groups of such a molecule may be represented as in reaction (i), Fig. 16a. In this reaction, two moles of periodate are consumed; one mole of formic acid and a stable dialdehyde (I) are produced. Oxidation of a reducing end group may be represented as in Fig. 16b. These reactions may be described as (ii) a normal Malapradian oxidation of α - glycol groups giving rise to a formyl ester (II); (iii) a hydrolysis of (II) yielding formic acid and an α - glycol structure (III); (iv) and (v) Malapradian oxidation of (III) in which formaldehyde and, in turn, formic acid are liberated with the formation of a malondialdehyde type structure (V) via structure (IV); (vi) oxidation of the activated hydrogen atom in (V) giving rise to structure (VI) which is susceptible to further oxidation by periodate. This oxidation of malondialdehyde is termed "overoxidation". The rate of overoxidation is greatly dependent on pH. and temperature (105); sodium metaperiodate buffered at

pH. 8 as oxidant at 35° provides optimum conditions for reaction (vi) and further oxidation to take place. Linear aldo-hexose polymers containing 1:3- and 1:4- glycosidic linkages produce the necessary active intermediate, and, under the above conditions, should be completely oxidised, the oxidation being a step-wise process from the reducing end-group. "Over-oxidation" of such linear polymers therefore results in the production of 1 mole of formaldehyde per monosaccharide residue. In the case of 1:6 - linked aldohexose residues, a malondialdehyde type structure is not produced (structure XI, reaction (vii), Fig. 16c); consequently step-wise degradation of such polysaccharides is inhibited whenever such a 1:6 - linkage is encountered in the chain. In a 1:3 - linked aldohexosan containing branch points at C_6, the linear 1:3 - linked chain is succeptible to overoxidation although the side chains are not; reactions (vii) - (ix), Fig. 16c.

The rate of overoxidation may be very greatly reduced by the use of potassium periodate as oxidant. Under these conditions, values for formaldehyde and formic acid release on total primary oxidation may be obtained; reactions (i) - (v), Fig. 16. Small errors due to overoxidation may be corrected by extrapolation of the linear portion of graph, moles product against time. Alternatively, a limited excess of sodium metaperiodate in the absence of sunlight may be used (cf. Section III). These oxidation conditions have been satisfactorily used in structural studies on laminarin (103) (108).

The results obtained from the periodate oxidation of the purified fractions of sample 1 and sample 2 are very similar. The formaldehyde liberated on complete primary oxidation was 0·026 (fraction D) and 0·029 (fraction 4) moles. On the assumption that each polysaccharide molecule

contains one normal free reducing group, and that each gives rise to one mole of formaldehyde, this corresponds to a D.P. of 40 - 36. The release of formic acid from the end-groups of a linear 1:3 - linked aldo-hexosan of D.P. 40 - 36 would be equivalent to 0·075 - 0·083 moles per anhydro-hexose unit. This is in good agreement with the experimental values obtained from both fractions of 0·078 - 0·083 moles. The periodate uptake (ca. 0·17 moles) is also in fair agreement with the theoretical value for such a linear molecule (0·13 - 0·14 moles).

However, on overoxidation the production of formaldehyde was 0·52 - 0·53 moles showing that a second type of linkage is present in the molecule. There are at least two possibilities, (a) an anomalous linkage may be present in the molecule, or (b) one half of the molecules may be terminated at the potential reducing end, by a 1:6 - linked hexose residue or some other polyhydric compound. The yield of formaldehyde was similar to that observed with laminarin; in this latter polysaccharide, ca. 50% of the molecules are terminated by 1:6 - linked mannitol residues (108). It was not, however, possible to detect mannitol by paper chromatography in the hydrolysate of Ochromonas Malhamensis polysaccharide.

This preliminary investigation has provided the first evidence for the presence of β - 1:3 - glucosidic linkages in a protozoal polysaccharide. This finding is of considerable biological interest, these linkages having been previously found only in polysaccharides isolated from the simpler organisms, e.g. glucan from bakers yeast (109), laminarin from brown algae (110), callose from grape vine (111) and pachyman from a Japanese fungus.(112).

Several structural features of the Ochromonas malhamensis polysaccharide have still to be determined. Periodate oxidation studies on the reduced polysaccharide would be highly informative. Methylation studies

would afford a measure of the average chain length and would give an indication of any branching or anomalous linkages. Finally, the location of the second sugar, which is as yet only tentatively identified as mannose by paper chromatography, could be investigated.

Conclusions

1. Ochromonas malhamensis synthesises a heterogeneous mixture of water-soluble polysaccharides containing glucose, galactose and mannose.

2. This mixture has been fractionated into components containing (i) galactose, glucose and a little mannose, and (ii) glucose and mannose.

3. The fraction containing glucose and mannose has been examined chemically. The proportion of mannose present could not be reduced below 11%. No evidence was obtained to show whether these sugars were present in the same molecule.

4. The optical rotation of the polysaccharide and the derived acetate indicates the presence of β - glycosidic linkages. Periodate oxidation studies showed that this linkage was predominantly 1:3. The products of enzymic and acid hydrolysis show the presence, in the molecule, of repeating β - 1:3 - glucosidic linkages.

5. The formaldehyde and formic acid production on total primary periodate oxidation suggest that one possible structure for the polysaccharide is an essentially linear molecule composed of ca. 40 β- 1:3 - linked anhydro-hexose units. However, the results of overoxidation with periodate indicate the presence of a second type of linkage, the identity and location of which is, as yet, unknown.

III. EXPERIMENTAL.

The samples of polysaccharide material were isolated by Dr. J. F. Ryley as follows:- pure culture of Ochromonas malhamensis were grown in daylight at 28°C. in 2 l. flasks containing 1·5 l. of aqueous medium pH. 6·0, 0·1% glucose, 0·1% "Oxoid" brand peptone and 0·1% Evans brand "Hepamino" liver extract The cells were harvested after 7 days and three volumes of ethanol were added to an aqueous extract.

Sample 1.

The material was stirred in water (1·5% solution) for 1 hour and the insoluble residue was then removed by centrifugation. The polysaccharides were precipitated by the addition of ethanol (3 vols.) redissolved in water, centrifuged and re-precipitated from solution with ethanol. This was repeated to yield a buff coloured material.

Found: reducing sugar content (as glucose) 79%; ash, 3·4%; protein nitrogen, 2·08%.

Paper chromatographic examination of the total acid hydrolysate (Solvent 1, Spray 1 and 2) showed that the monosaccharides present had the same chromatographic mobility and colour reaction with aniline oxalate as the reference sugars D - galactose, D - glucose, D - mannose, and D - xylose. The amount of glucose in the hydrolysate was greater than that of galactose or mannose which were present in approximately equal proportions. Xylose was present in trace amounts only.

Periodate Oxidation.

20 Ml. of solution containing 2·89 m.g. polysaccharide / ml. (concentration determined by acid hydrolysis) were added to 3·5% sodium metaperiodate solution (6·7 ml.) and the mixture placed, together with a reagent blank, in the dark at 2°C.

Aliquots (5 ml.) were removed at intervals into ethylene glycol and titrated against 0·01177 N - sodium hydroxide. The corrected titre, which was constant at 2 - 4 days was 0·46 ml. corresponding to a formic acid production of 0·081 moles.

The periodate uptake (method 3) after 4 days oxidation was 0·34 moles.

Attempted Deproteinisation.

The material was dissolved in 0·1 M - sodium chloride solution and shaken mechanically in a stoppered glass bottle with freshly distilled toluene. After 24 hours the solution was centrifuged. The precipitated protein collected at the water-toluene interface and the aqueous solution was removed by suction. The deproteinisation was repeated 12 times. The opalescent aqueous solution, which was yellow-brown in colour, was then precipitated with ethanol (3 vols.).

Found:- protein nitrogen, 0·89%.

Cetavlon Precipitation.

The material (4·0 g.) was dissolved in water (200 ml.) and 3% Cetavlon solution (100 ml.) added slowly with stirring. A brown precipitate was removed by centrifugation and to the supernatant solution ethanol (3 vols.) was added. Cetavlon was removed from the precipitate by a further six ethanol precipitations to yield a white powder (2·29 g.).

Paper chromatographic examination of the total acid hydrolysate showed that a little of the galactose had been removed; the proportions of glucose, mannose and xylose present were the same as before.

Found:- reducing sugar content (as glucose), 90%; ash., 1·9%; protein nitrogen, 0·75%; $[\alpha]_D$ + 15° (C, 0·3 in water).

Partial acid hydrolysis.

The polysaccharide (10 m.g.) was heated at 100°C. in 0·5N - sulphuric acid for 1·5 hours. Chromatographic examination (Solvent 1, 2; Spray 1, 2) showed the presence, in addition to glucose, galactose, mannose, and traces of xylose, of sugars having the same mobilities as standards of laminaribiose, laminaritriose and laminaritetraose. The R.g. values of these sugars were

(a) in solvent 1:- 0·75, 0·52, 0·33, 0·23,

(b) in solvent 2:- 0·81, 0·56, 0·38, 0·28.

R.g. values (solvent 2) quoted by Peat, Whelan and Lawley (113) were:- laminaribiose, 0·80; laminaritriose, 0·57; laminaritetraose, 0·40; laminaripentaose, 0·28.

Qualitative enzymic hydrolysis.

A solution of the polysaccharide (0·5 ml., 10 m.g.) was incubated for 2 days with a β - glucosidase preparation (10 m.g.) from Cladophora rupestis prepared by Dr. W. A. M. Duncan. Chromatographic examination as before showed the presence of glucose, mannose, laminaribiose, laminaritriose, laminaritetraose, and higher sugars. Similar hydrolysis of laminarin gave oligosaccharides having identical mobilities.

On similar incubation with almond emulsin, prepared by Dr. F. B. Anderson, only faint traces of glucose could be detected.

Attempted purification.

The following methods were used:-

(a) Copper complexing.

Fehlings solution (5 ml.) was added to an aqueous solution of the polysaccharide (10 ml., 180 m.g.) and the mixture stirred for 1 hour and allowed to stand. No precipitation occurred.

(b) Cetavlon precipitation at varying pH. values.

A sample of the polysaccharide (100 m.g.) was dissolved in a solution (90 ml.) containing 0·2 M- acetate buffer (10 ml.), pH. 5·0. 0·2 M - Borate buffer was then added slowly with stirring. No precipitation occurred until the pH. of the solution reached 9·0. At this pH. a considerable precipitate formed which was removed by centrifugation, dissolved in water and re-precipitated with ethanol. Ethanol was then added to the remaining mixed buffer solution and the resulting precipitate collected by centrifugation, dissolved in water, and re-precipitated. Both fractions were then hydrolysed and examined chromatographically (Solvent 1, Spray 2). The proportions of monosaccharides present were the same.

(c) Acetone fractionation.

Acetone was added slowly, with stirring, to a solution of the polysaccharide in water (100 m.g., 50 ml.). Precipitates were removed at intervals, hydrolysed, and examined chromatographically.

Volume of Acetone (ml.)	Approximate proportions of sugars present (visual estimation)
50	Galactose, glucose, mannose (2:1:1)
80	Glucose, mannose (5:1) trace xylose
150	Glucose, mannose (5:1) trace xylose.

Estimation of the amount of each sugar present in mixtures of glucose and mannose.

An aqueous solution of glucose (10 ml., 20·1 m.g.) was mixed with mannose solution (3 ml., 5 - 6 m.g.) in a standard flask (25 ml.). A solution (5 ml., 75 m.g.) of a commercial preparation of glucose oxidase ("Deeo" enzyme system, Takamine Co.) was then added and the solution diluted to 25 ml. with water and incubated at 35°C. Samples (ca. 6 ml.) were removed at intervals and heated in a stoppered centrifuge tube, at 100°C. for 3 minutes. The cooled solution was then shaken and the precipitated protein removed by centrifugation. The reducing sugar content of aliquots (2 ml.) of the supernatant solution was determined by the Somogyi reagent.

Results.

TABLE 23

Time	Titre Difference	$T.D._o - T.D._t$	Glucose destruction
(hr.)	(ml.)	(ml.)	%
0	7·07	-	-
3	3·03	4·04	72
5	2·66	4·41	79
30	1·24	5·83	104
48	1·22	5·85	104

Chromatographic examination of the solution after 48 hours (Solvent 1, Spray 2) showed that mannose was the only reducing sugar present.

A similar oxidation mixture was set up containing glucose (20·1 m.g.) and mannose (2·36 m.g.).

Results.

TABLE 24

Time (hr.)	Titre Difference (ml.)	$T.D._o - T.D._t$ (ml.)	Glucose destruction %
0	6·29	-	
12	3·11	3·18	56
30	0·56	5·73	102
48	0·55	5·74	102

The amount of each sugar present, determined by titration after thirty hours oxidation is thus:-

glucose, 20·5 m.g. (102% theoretical)

mannose, 2·26 m.g. (96% theoretical)

Acetone fractionation.

The polysaccharide (2·0 g.) was dissolved in water (700 ml.) containing approximately 0·5 g. ammonium acetate. Acetone was added slowly with stirring. The precipitates were removed by centrifugation, dissolved in water, re-precipitated and dried with alcohol and ether. A portion of each precipitate was hydrolysed and examined chromatographically: the weight of each fraction and the sugars present in the hydrolysate are shown below.

	Volume of acetone added (ml.)	Weight of fraction (m.g.)	Monosaccharides present
(1)	800	445	galactose (+++), glucose (++), mannose (++).
(2)	1000	771	glucose (++), mannose (+) galactose (±)
(3)	2800	723	glucose (++), mannose (+), xylose (±).

Precipitate (3) was redissolved in water (200 ml.) and precipitated with acetone (600 ml.) to yield a material (400 m.g.) which was free from xylose. (Precipitate (3A)).

The reducing sugar contents of fractions (2) and (3A) were then determined by total acid hydrolysis and subsequent destruction of the glucose using glucose oxidase as described above.

Weight of Fraction	Titre Difference at (hrs.) 0	30	48
Fraction 2			
(22·94 m.g.)	5·71	0·67	0·71
Fraction 3			
(24·99 m.g.)	6·81	0·77	0·80

Sample (2) thus contains 71·0% glucose and 11·1% mannose. Sample (3A) contains 77·9% glucose and 11·3% mannose.

These two fractions were combined and dialysed (3 days) against distilled water. (A control experiment had previously shown that 98% of the polysaccharide was retained on dialysis). A slight brown precipitate was removed by centrifugation followed by filtration through an asbestos pad. The solution was then precipitated with ethanol to yield 770 m.g. material - fraction (4).

On analysis as above, this fraction was found to contain 84·7% glucose and 12·2% mannose. The reducing power, determined by the Somogyi reagent, corresponded to 1 reducing group per 63 anhydro-glucose residues (laminaribiose standard).

Sodium metaperiodate oxidation of fraction (4).

Fraction (4) (249·0 m.g.) was dissolved in water and diluted to

25 ml. in a standard flask. The optical rotation of this solution was measured:-

$$[\alpha]_{D}^{20} + 10{\cdot}8^{\circ} \text{ (C., 1·0; 1, 2)}$$

An aliquot (20 ml.) of the above solution was placed in a standard flask (100 ml.). 6·4% sodium metaperiodate solution (4 ml.) was added and the mixture diluted to 100 ml. This solution, together with a reagent blank, was placed in the dark at 2°C. Aliquots were removed at intervals and analysed as below.

(a) Formic acid release.

Aliquots (10 ml.) were withdrawn into ethylene glycol and titrated with 0·0102N - sodium hydroxide.

TABLE 25

Time (days)	Titre (ml.)	Blank (ml.)	Corrected Titre (ml.)	Formic Acid (moles)
0	0·04	0·00	0·00	-
2	0·72	0·00	0·68	0·0580
7	0·98	0·00	0·94	0·0802
9	1·04	0·00	1·00	0·0853

The average value of the production of formic acid after 7 - 9 days oxidation corresponds to 1 mole per 12 hexose units.

(b) Periodate uptake.

Aliquots (5 ml.) were removed at 7 and 9 days and the periodate uptake determined by titration with 0·0965N - iodine solution. The titre differences were 0·20 and 0·23 ml. corresponding to a periodate uptake of 0·16 and 0·18 moles respectively.

(c) Formaldehyde release.

Aliquots (2 ml.) were analysed by the ferricyanide method (method 5). The absorption value, which was constant between 7 and 9 days, corresponded to a formaldehyde production of 0·029 moles, i.e. 1 mole per 34·5 hexose residues.

In a second experiment using 195·1 m.g. of fraction 4, the oxidation was allowed to proceed in the dark at 2°C. for 6 days. Aliquots were analysed and the oxidation mixture was then stored in the dark at room temperature for a further 2 days.

Results.

Time (days)	3	6	8
Formic acid (moles)	0·063	0·078	0·081
Periodate uptake (moles)	0·13	0·17	0·18

The average value of the formic acid release at 6 - 8 days corresponds to 1 mole formic acid per 12·6 hexose residues.

Overoxidation of fraction 4.

A solution of fraction 4 (2 mls., 9·68 m.g.) was treated at 35°C. with 0·3 M- sodium metaperiodate solution (2 ml.) and 0·1 M - phosphate buffer (12 ml., pH. 8) in a total volume of 25 ml. A reagent blank was also prepared. Samples (2 ml.) analysed at intervals showed that the production of formaldehyde (estimated by method 5) was constant after oxidation for 18 hours and amounted to 0·53 moles per anhydro-glucose unit. The residual polysaccharide was stable, even after 4 days oxidation, under these conditions.

Sample 2

Purification and Acetone Fractionation.

This sample (8 g.) was purified by aqueous extraction and Cetavlon precipitation as before to yield a white powder (5·6 g.). This was dissolved in water (1 l.) containing ammonium acetate (0·5 g.). Acetone was added slowly with stirring. The resulting precipitates were collected by centrifugation, dissolved in water, filtered through an asbestos pad and reprecipitated with alcohol. Portions of the dried precipitates were then hydrolysed and examined chromatographically.

Volume of Acetone (ml.)	Fraction	Weight (g.)	Sugars present in hydrolysate
1,200	A	1·908	Galactose, glucose, mannose.
1,600	B	2·897	Glucose, mannose, trace galactose.
3,500	C	0·311	Glucose, mannose, trace xylose.

Proportion of glucose and mannose in the fractions.

The amounts of glucose and mannose (or mannose plus galactose) were determined as above:-

Fraction A contained 56·9% glucose and 11·7% galactose plus mannose (estimated as mannose).

Fraction B contained 73·5% glucose and 8·6% mannose.

Fraction C contained 62·1% glucose and 11·3% mannose.

Preparation and fractionation of the acetate.

Fraction B (500 m.g.) was dispersed in formamide (5 ml.) in a round bottomed flask (50 ml.). Pyridine (10 ml.) and acetic anhydride (4 ml.) were added and the mixture shaken in the dark for 4 days. The solution was then poured into ice-water (500 ml.) with stirring. After 2 hours, the supernatant solution was removed by centrifugation. The acetate was stirred with water (200 ml.) which was again removed by centrifugation. The acetate was washed with water a further three times and finally collected on a sintered glass crucible. Adhering water was removed by suction and the acetate was dried over phosphorous pentoxide. Chloroform (100 ml.), in which the dry acetate was readily soluble, was then passed through the crucible under gentle suction and the acetate recovered by precipitation with petroleum ether (B.P. 80 - 100°C., 3 vols.). Yield, 480 m.g., $[\alpha]_D$, - 59.3° (C. 0.9, in chloroform).

A portion of the acetate (29.00 m.g.) was added to water (3 ml.) and acetone (15 ml.) in a conical flask (100 ml.). After solution of the acetate by gentle warming, 0.0500 N-sodium hydroxide (15 ml.) was added and the mixture shaken for 36 hours. 0.05 N - Sulphuric acid (15 ml.) was then added and the solution back titrated with 0.0500 N- sodium hydroxide (phenolphthalein indicator). A similarly treated reagent blank was titrated in the same way.

Results.

Corrected titre = 5.55 ml.

$$\text{Percentage acetyl groups} = \frac{5.55 \times 0.050 \times 4.3}{29.00}$$

$$= 41.2\%.$$

The acetate (400 m.g.) was dissolved in chloroform (20 ml.) and

petroleum ether (B.P. 80 - 100°) was added slowly with stirring. Fractions were collected after the addition of 30 ml. (240 m.g.) and 60 ml. (140 m.g.) of petroleum ether.

The fractions were then dissolved in 50 - 50 mixtures of dry chloroform and dry methanol (10 ml.) and a solution of barium oxide in dry methanol (2 - 3 drops) added. The solutions were left at 2°C. for 24 hours. Two drops were then withdrawn into water (0·5 ml.) containing phenolphthalein indicator. Both solutions gave an alkaline reaction showing that barium oxide was in excess. The precipitated polysaccharides were collected by centrifugation, dissolved in water and precipitated with ethanol. Samples of each fraction were incubated with glucose oxidase as before. The amounts of glucose and mannose present were:-

Fraction 1: 9·5% mannose, 82·1% glucose.

Fraction 2: 9·7% mannose, 80·9% glucose.

Re-fractionation of fraction B.

Fraction B (2·0 g.) was dissolved in water (500 ml.) containing a little ammonium acetate. Acetone was added as before and the resulting precipitates collected by centrifugation.

Volume of Acetone (ml.)	Fraction	Yield (g.)	% Glucose	% Mannose
750	B	0·23	73·6	8·5
1500	B	1·24	78·2	8·8

Fraction B was dissolved in water and dialysed for 3 days against distilled water. The solution was then filtered through an asbestos pad and precipitated with ethanol to yield a white powder (1·05 g.) hereafter referred to as fraction D.

Found:- glucose content, 85·1%; mannose content, 10·2%;

$[\alpha]_D^+$ 9·1 (C. 0·4 in water); reducing power, 63.

Periodate Oxidation of Fraction D.

(1) Oxidation with potassium metaperiodate.

To a solution (20 ml.) containing 183·7 m.g. fraction B were added 5% potassium chloride solution (15 ml.) and 0·3 M - sodium metaperiodate solution (20 ml.). The mixture was shaken in the dark at room temperature. Aliquots (5 ml.) were removed at intervals and the formic acid production measured as before.

Results.

TABLE 26

Time (days)	Corrected Titre (ml.)	Formic Acid (moles)
3	0·80	0·0785
5	0·92	0·0908
7	0·96	0·0950
9	1·01	0·0998

Extrapolation of the linear part of the graph (Fig. 15) gives a value for the formic acid release on total primary oxidation of 0·078 moles. This corresponds to 1 mole per 12·8 hexose residues.

(2) Sodium metaperiodate oxidation.

0·3 M - Sodium metaperiodate solution (8 ml.) was added to a solution containing 195·7 m.g. fraction D. The solution was diluted to 100 ml. with water and placed, together with a reagent blank, in the dark at room temperature. Aliquots were analysed at intervals.

Results:-

TABLE 27

Time (hrs.)	2	5	24	48
Formic Acid release (moles)	0·0386	0·0574	0·0817	0·0855
Periodate uptake (moles)	-	-	0·153	0·169
Formaldehyde release (moles)	-	0·017	0·025	0·027

Extrapolation of the linear part of the graph (Fig. 15) gives a value for the formic acid release of 0·078 moles, i.e. 1 mole per 12·8 hexose residues.

Overoxidation

On oxidation at pH. 8·0 with excess sodium metaperiodate, 0·52 moles formaldehyde were liberated per anhydro-hexose residue.

Attempted Detection of Non-Reducing Sugars.

(1) A total acid hydrolysate of fraction D (10 m.g.) was examined, after neutralisation with barium carbonate, by paper chromatography (solvent 3, spray 3). Glucose and mannose were the only substances which could be detected. On similar examination of laminarin, mannitol was readily detected (108).

(2) A similar neutralised acid hydrolysate (1 ml., ca. 5 m.g.) was incubated with glucose oxidase (ca. 25 m.g.) at 37° C. for 48 hours. On chromatographic examination (solvent 1 and 3, sprays 1, 2 and 3) of the solution after deproteinisation, mannose was the only substance which could be detected. This experiment characterises the major monosaccharide component as D-glucose.

SECTION VII

STUDIES OF THE TRANSGLYCOSYLASE ACTIVITY OF CELL - FREE EXTRACTS OF TETRAHYMENA PYRIFORMIS.

I. INTRODUCTION.

Tetrahymena pyriformis is a free living ciliate commonly found in fresh water ponds. The first bacteria-free culture of T. pyriformis (then known as Glaucoma pyriformis) was isolated by Lwoff in 1923 (114). Since then, and especially since it was found possible to grow cultures of T. pyriformis in synthetic media (115) (116), the metabolism of the organism has been extensively investigated. Such studies are also facilitated by the high rate of growth and reproduction. Several review articles have been published on the metabolism, growth, nutrition, specific enzyme activity, and genetics of this ciliate (117) (118) (119) (120) (121). It has been shown to synthesise, and to store as a food reserve, large quantities (ca. 22% on a dry weight basis) of a polysaccharide which is closely similar to a typical animal glycogen (15). Ryley has investigated the enzyme systems responsible for the synthesis of this polysaccharide (16); cell free extracts of the organism were found to possess succinic and lactic dehydrogenases, aldolase, hexokinase, oxoisomerase, phosphoglucomutase and phosphorylase activities indicating that the synthesis of glycogen may take place as shown in Scheme 1 (p. 10). Amylase and maltase activities were also detected.

Recent investigations have shown that a number of enzymes which normally catalyse the hydrolytic or phosphorolytic breakdown of carbohydrates can, under certain conditions, catalyse the transfer of a monosaccharide residue from a carbohydrate molecule to an acceptor. In the present work, it was found that one such transferase system was present in cell free extracts of Tetrahymena pyriformis; an account of an examination of this system is given in this section.

This type of reaction may be represented by the general equation:-

$$D - O.R + H - O.A \rightleftharpoons D - O.A + H - O.R \quad \ldots$$

Donor substrate Acceptor substrate ... equation 1.

where D represents the residue which is transferred.

The molecule H - O.A may be an alcohol or a carbohydrate. Hydrolases may also be regarded as members of a group of transferring-enzymes having highly specific requirements for the acceptor substrate which, in this case, is water.

Miwa and Takano, using cell free extracts of green leaves, were able to effect the transfer of a glucose residue from an aryl glucoside to an alcohol (122) (123). In the course of purification studies they observed that the hydrolase and transglycosylase activity appeared to be due to the same enzyme. It seems probable that in many cases these activities are due to the same enzyme, the final equilibrium being dependant on the concentration of reactants.

Rabaté (1935) was the first to recognise that certain hydrolases could catalyse reactions of the above type (equation 1) where D is a glycosyl residue and R and A are alkyl or aryl radicals, or a chain of one or more sugar residues (124). These enzymes have been referred to as "transferases",

"transglycosidases" or "transglycosylases". It was originally believed that the reaction involved the transfer of a glycosidic (D.O.) radical. It has, however, been shown by isotopic studies with e.g. sucrose phosphorylase (125), invertase (126) and almond emulsin (127) that the transfer involves the glucosyl (D) and not the glucosidic (D.O.) radical. The term "transglycosylase" is therefore now used to describe such enzymes.

It is of interest to note that, in most reactions which proceed according to equation 1, the configuration of the glycosidic linkage is unaltered. For example, sucrose phosphorylase reversibly transfers a glucosyl unit from α- glucose - 1 - phosphate to fructose, forming sucrose, in which the glucose is still in the α- form. If the group D.O.R. is split into D + O.R. as the result of a nucleophilic attack on D.O.R. it would be expected that inversion of the configuration (Walden inversion) would result. It has, however, been shown that some substitution reactions of this type which proceed with retention of configuration do, in fact, consist of two successive reactions both of which involve Walden inversions (128) (129). A similar mechanism, as shown in equations 2 and 3, has been suggested to account for the retention of configuration in transglycosylation reactions (130) (131) (132) (133).

$$D - O.R. + H - Enzyme \rightleftharpoons D - Enzyme + H - O.R. \quad \text{.... equation 2}$$

$$D - Enzyme + H - O.A. \rightleftharpoons D - O.A. + H - Enzyme \quad \text{.... equation 3.}$$

The synthesis of oligosaccharides has been reported from maltose (134) (135) (136) (137) (138) (139), sucrose (140) (141) (142), isomaltose (137) (143) and cellobiose (137) (144) (145) (146). In similar syntheses, using disaccharides as donor substrate and amino sugars or pentoses as acceptor molecules (146) (147) (148), the resulting glucosyl derivatives have

been identified by chromatographic and chemical methods.

Stacey (147) has suggested that plant gums and dextrans may be formed *in vivo* by the cross-linking of oligosaccharides synthesised by transglycosylation reactions. It is possible also that the maltosaccharides required as primers for phosphorylase action (149) (150) (151) and as substrate for D - enzyme, which is itself a transglycosylase (152) (153) (154), are synthesised in this manner.

This section contains an account of the qualitative demonstration of hydrolase and transglycosylase activities in cell-free extracts of *T. pyriformis* and of the separation, on a large scale, of the oligosaccharides synthesised from maltose by such extracts.

The specificity requirements of the enzyme system for donor and acceptor substrates has been investigated by incubation of the cell free extract with concentrated solutions of various individual mono- and di-saccharides and with concentrated solutions containing equal quantities of maltose and various monosaccharides.

II. DISCUSSION.

A pure culture of Tetrahymena pyriformis was obtained from Dr. Ryley and large scale cultures prepared, the cells being harvested after 6 days growth. Freeze-drying of the cells in 0·1M - citrate buffer pH 6·0 disrupted the cell membrane and cell-free extracts were prepared by centrifugation of an aqueous extract of the freeze-dried cells. These extracts were then freeze-dried to provide the "enzyme powders" used in the subsequent experiments. Chromatographic examination of aqueous solutions of such powders showed that a trace of glucose was the only reducing sugar present; the amount of this did not increase on autolysis indicating that any glycogen present was completely hydrolysed during the aqueous extraction of the freeze-dried cells.

Portions of the enzyme powder were incubated with dilute solutions of various carbohydrates. It was found that maltose, isomaltose, panose, maltotriose, methyl - α - glucoside, glucose - 1 - phosphate, cellobiose, sucrose and starch were readily hydrolysed but that lactose was not attacked. In view of the considerable α - glucosidase activity of the extract it was considered probable that the enzymes present would catalyse the transfer of glucose from α - linked glucosides and oligosaccharides to various acceptor substrates. Initial experiments using glucose itself as the donor substrate failed to produce transglucosylation to other glucose molecules, i.e. to effect the enzymic polymerisation of glucose. In this respect the T. pyriformis enzymes differ from those of almond emulsin (155) and Aspergillius niger (156) which can synthesise disaccharides from glucose.

From this it would appear that any transferase activity present in the T. pyriformis extract does not effect the addition of glucose molecules to the acceptor via a glucose-enzyme complex intermediate but rather that the latter will be of the glucosyl-enzyme type.

Enzymic Synthesis of Oligosaccharides by Transglucosylation from Maltose.

Oligosaccharides containing α - 1:4 and α - 1:6 glucosidic linkages have been produced by transglucosylation using maltose as substrate by various enzyme preparations as shown in Table 28.

TABLE 28

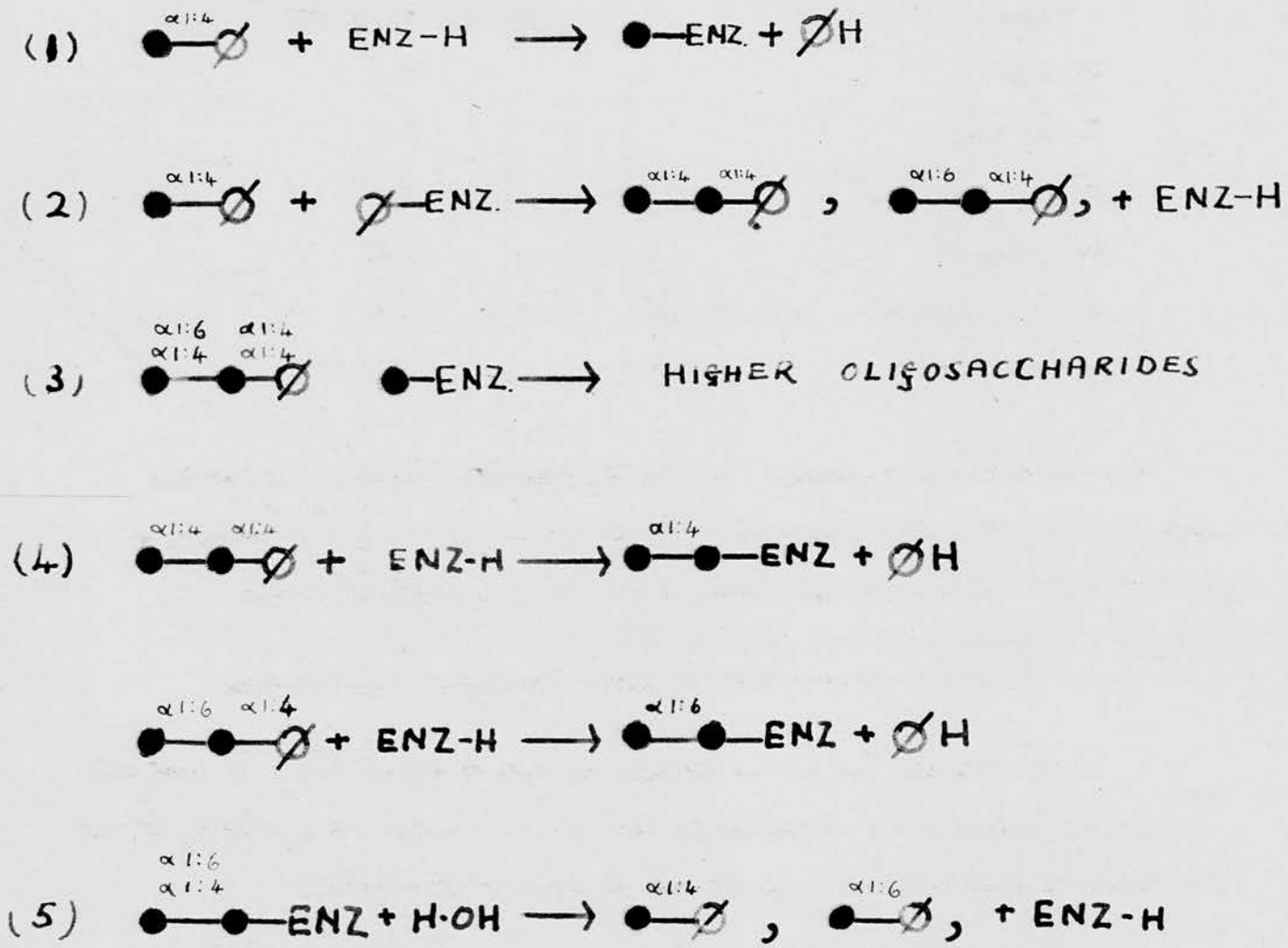
Biological source	Oligosaccharides	Reference
Pencicillium chrysogenum Q 176	Isomaltose, isomaltotriose, panose, 6^2 - α - isomaltosylmaltose	(136)
Aspergillus niger (strain 152)	Isomaltose, isomaltotriose, panose.	(138)
Aspergillus oryzae	Isomaltose, isomaltotriose, panose, 6^2 - α - isomaltosylmaltose.	(134)
Escherichia coli	Maltotriose, higher oligosaccharides	(135)
Rat liver	Maltotriose, maltotetraose	(139)
Brewers yeast	Isomaltose, maltotriose, panose	(157)
Cladophora rupestis	Isomaltose, maltotriose, panose, 6^3 - α - glucosyl-maltotriose, maltotetraose.	(137)
Tetrahymena pyriformis	Isomaltose, maltotriose, panose, 6^3 - α - glucosyl-maltotriose.	

In the present investigation a cell free extract of T. pyriformis

(hereafter called T.P. enzyme) was examined qualitatively for transglucosylase activity using maltose as both the donor and the acceptor substrate. The disaccharide (as a 20% solution) was incubated with the enzyme preparation at 35°C. at pH. 6·0 (by virtue of the buffer present in the enzyme powder); samples were removed at intervals and the reducing sugars present examined by paper chromatography. After two days incubation, in addition to glucose and maltose, carbohydrates having the same mobilities as maltotriose and panose were present together with traces of other sugars which had lower mobilities than maltotriose. After four days incubation, a faint trace of a sugar having the same mobility as isomaltose was present together with increased quantities of maltotriose and panose. It was found that the enzymic synthesis reached a maximum after ca. 10 days at which time large quantities of glucose, maltose, isomaltose, maltotriose and panose were present. A small proportion of sugars having lower chromatographic mobilities (Table 30, p.122) were also detected. The oligosaccharides were not synthesised by purely chemical means since they were not produced in an identical digest containing glucose and maltose together with heat-inactivated enzyme.

In order to identify the products of enzyme action and hence postulate a possible reaction mechanism, a large scale digest (containing ca. 30 g. maltose; 20% solution) was prepared. The enzymic action was terminated after 10 days incubation and the sugars separated by use of a charcoal - Celite column. Table 29 gives the approximate yield and identity of the oligosaccharides produced.

FOSSIBLE MECHANISM FOR TRANS - α - GLUCOSYLATION FROM MALTOSE



● & Ø represent a glucosyl group and a reducing glucose residue, respectively;

ØH represents glucose;

α1:6 represents isomaltose, etc.

Fig 17

TABLE 29

Sugar	Approx. Yield (%)
Maltose	11·5
Isomaltose	5·7
Panose	9·8
Maltotriose	10·8
6^3 ∝ - glucosyl - maltotriose[+]	1·8
Unidentified ϕ	1·5

[+] Nomenclature as recommended by the Biochemical Journal; systematic name, O - ∝ - D - glucopyranosyl - (1→6) - O - ∝ - D - glucopyranosyl - (1→4) - O - ∝ - D - glucopyranosyl - (1→4) - D - glucopyranose.

ϕ Mainly mixtures; examination of these fractions is proceeding.

It is apparent, since the amounts of panose and maltotriose produced are closely similar, that transfer to the hydroxyl groups at C_4 and C_6 of the non-reducing glucose residue in maltose is equally favourable.

From the products and from the observation that isomaltose was produced only after relatively large amounts of maltotriose were present in the digest a possible reaction mechanism may be as represented in Fig. 17. This can be described as follows:- (1) maltose, acting as the donor substrate, is attacked by the enzyme and forms glucose and an ∝- glucosyl-enzyme complex (assuming the reaction involves a Walden inversion); (2) unattacked maltose may then act as an acceptor molecule, with active acceptor sites at the hydroxyl groups attached to carbon atoms 4 and 6 of the non-reducing glucose residue, thus giving rise to maltotriose or panose; (3) higher oligo-saccharides may be formed by the transfer of glucosyl residues

to the non-reducing end of these trisaccharides; (4) maltose and isomaltose may be formed by the hydrolysis of the trisaccharides and higher oligosaccharides; these disaccharides may then in turn act as acceptor molecules.

Alternatively, the disaccharides may be produced by transfer of a glucosyl radical to the hydroxyl groups at C_4 and C_6 of glucose. In this case, the enzyme system must be able to utilise either glucose or glucosaccharides as acceptor substrates; the slow production of isomaltose may be explained by the glucose concentration being low in the early stages of the reaction compared to that of maltose. Buston and Khan (158) found that, on incubation of cell-free extracts of Chaetomium globosum with cellobiose, disaccharides were not produced until appreciable quantities of cellotriose was present; these authors suggested that the disaccharides might be formed by hydrolysis of the trisaccharides.

From the relative amounts of isomaltose, maltotriose and panose produced, it appears that the hydroxyl groups at carbon atoms 4 and 6 in a glucose molecule or anhydro-glucose residue are equally favourable sites for addition of a glycosyl residue. It is not yet known whether such syntheses are due to one or two enzymes.

Transglucosylation with Various Donor Substrates.

It has been shown that extracts of Cladophora rupestis may effect transglucosylation with either maltose or cellobiose as substrate (137) (163). In the present work concentrated solutions of various carbohydrates were incubated with T.P. enzyme (Table 33, p. 130). No synthesis occurred with glucose, xylose, sucrose or glucose - 1 - phosphate. With maltotriose and panose the oligosaccharides formed were similar to those produced from maltose. With isomaltose, however, glucose, isomaltose, isomaltotriose

and small amounts of higher sugars were the only sugars which could be detected after 5 days incubation; this indicates that the synthesis occurs by transfer of a glucosyl group to isomaltose and not to glucose since in the latter case considerable quantities of maltose would be formed. On incubation of methyl - α - glucoside with T.P. enzyme only traces of maltosaccharides were formed. With cellobiose, however, several sugars having low chromatographic mobility were formed. This latter synthesis proceeded at a lower rate than those using maltosaccharides as donor substrate, but after 11 days incubation appreciable quantities of three new oligosaccharides were present. The chromatographic mobilities of these did not correspond to any of the maltosaccharides or to laminaribiose, gentiobiose, cellotriose or gentiotriose. Further examination of these sugars has not been attempted.

Trans-glucosylation with Maltose as Donor Substrate and Various Monosaccharides as Acceptor Molecules.

It has been found that transglucosylation may be effected from cellobiose to various pentose acceptor molecules using enzyme preparations from moulds and seaweeds (145) (159). With seaweed enzymes, however, such transfer did not occur using maltose as the donor substrate.

In the present work, T.P. enzyme has been incubated with concentrated solutions containing equal quantities of maltose and various monosaccharides. With each of D - xylose, D - lyxose, D- ribose and L - arabinose, sugars were produced, in addition to those formed from maltose alone, which had lower chromatographic mobilities than glucose and which formed pink "spots" on treatment with the aniline oxalate spray reagent (spray 2). On incubation of maltose and galactose the sugars formed were indistinguishable from those formed from maltose alone, with the exception of one oligosaccharide

which had R.g. value 0·32. It is apparent, therefore, that the transferase system has a fairly low specificity for the acceptor molecule. No transference to fructose could, however, be observed.

From these results it is apparent that the molecule H - O. A. in the general equation

$$D - O.R. + H - O.A. \rightleftharpoons D - O.A. + H - O.R.$$

may, where D.O.R. is maltose, be an oligosaccharide, a monosaccharide or water, depending on the concentration.

Conclusions.

1. Cell free extracts of Tetrahymena pyriformis possess considerable transglycosylase activity.

2. These extracts catalyse the transfer of a glucosyl residue from maltose, isomaltose, maltotriose or panose, to glucose, various pentoses, maltosaccharides or water.

3. Cellobiose may also act as a donor substrate.

III. EXPERIMENTAL.

A pure culture of Tetrahymena pyriformis, kindly provided by Dr. Ryley, was maintained by periodic subinoculation under sterile conditions into plugged (cotton wool) test tubes containing 10 ml. of an aqueous (tap water) medium containing 1% "Oxoid" bacteriological peptone and 0·4% sodium chloride, which had been adjusted to pH. 7·3 and sterilised by autoclaving at 120°C. for 45 minutes. The tubes containing the cultures were stored at room temperature and were subcultured at fortnightly intervals.

Large Scale Culture.

Portions (400 ml.) of medium prepared as above were placed in conical flasks (2 l.). The flasks were plugged with cotton wool and sterilised in the autoclave (120°C., 45 minutes). The cooled flasks were then inoculated, by means of a sterilised Pasteur pipette, with 2 - 3 drops of a 8 - 10 day old test-tube culture. The inoculated flasks were placed in the dark at 30°C. Under these conditions the culture density reached a maximum after 6 days growth. After 12 days few live cells could be detected.

Preparation of cell-free extracts.

Twenty-two flasks were incubated as above for 6 days; the contents were combined, and the cells harvested using a Sharples supercentrifuge. The resultant sludge was dispersed in 0·1 M - citrate buffer pH. 6·0 (150 ml.)

and freeze dried to yield 7 g. of a yellow-brown powder. Microscopic examination of an aqueous smear of this material showed that the cells had disintegrated. Part of the freeze dried powder (0·35 g.) was added to water (10 ml.) and allowed to stand, with frequent stirring, at room temperature for one hour. The mixture was then centrifuged at 2°C. (5,000 r.p.m.). Decantation yielded a yellow opalescent solution (7 ml.) which was found, by microscopic examination, to be free from cells and cell particles.

The enzymically active powders examined in the subsequent work were prepared by extraction of the freeze dried cells (ca. 7 g.) with water (200 ml.) and freeze drying of the resultant cell free extract (yield of product, ca. 4·8 g.). Qualitative experiments showed that the hydrolytic activity of one such powder, which had been stored over calcium chloride at 2°C., was not appreciably diminished after 1 year. In all experiments described below, however, the enzyme powders used were no more than 4 weeks old.

Autolysis of the Enzyme Preparation.

Enzyme powder (20 m.g.) was dissolved in water (0·5 ml.). Samples were examined chromatographically (Solvent 1, spray 1) at intervals. A faint trace of glucose was the only reducing sugar which could be detected; the amount present after 5 days incubation at 35°C. did not appear to be greater than that present at zero time.

Carbohydrase Activity of the Extract.

Enzyme powder (20 m.g.) and carbohydrate (20 m.g.) were dissolved in water (1 ml.) and incubated at 35°C. for 24 hours. Samples were then examined chromatographically (Solvent 1, spray 1). The following sugars

were hydrolysed:- maltose, isomaltose, panose, maltotriose, starch, cellobiose, sucrose, methyl - ∝ - glucoside, glucose - 1 - phosphate. Lactose was not attacked.

Enzymic Action with Glucose as the Sole Substrate.

A digest consisting of glucose (200 m.g.) and enzyme powder (20 m.g.) dissolved in water (1 ml.) was incubated at 35°C. Samples were examined chromatographically as above. Glucose was the only reducing sugar present up to 15 days incubation.

Enzymic Synthesis of Oligosaccharides by Transglucosylation from Maltose.

A digest containing maltose (200 m.g.) and enzyme powder (20 m.g.) dissolved in water (1 ml.) was prepared; toluene was added to maintain asceptic conditions. The digest was incubated at 35° together with a control consisting of maltose (200 m.g.) in water (1 ml.). Samples of the digest and the control were examined at intervals, by paper chromatography (Solvent 1, spray 1). No reducing sugar other than maltose was observed in the control. The enzyme digest was found to contain several reducing sugars as shown in Table 30.

TABLE 30

Incubation Time (days)	R.g. values (solvent 1) and relative amounts of reducing sugars present.
0	0 62 (+++)
2	1·0 (++); 0·62 (+++); 0·39 (+); 0·28 (+); others (t)
4	1·0 (++); 0·62 (+++); 0·47 (t); 0·39 (t); 0·28 (+); others (t)
6	1·0 (++); 0·62 (+++); 0·47 (+); 0·39 (+); 0·28 (+); others (t)
8	1·0 (+++); 0·62 (+++); 0·47 (+); 0·39 (++); 0·28 (++); others (+).
10	1·0 (+++); 0·62 (+++); 0·47 (++); 0·39 (++); 0·28 (++); others (+)
15	1·0 (+++); 0·62 (+++); 0·47 (++); 0·39 (++), 0.28 (++); others (+).

+++, ++, +, and t indicate intense, strong, moderate and weak "spots" respectively.

(2) Large Scale Digest.

Substrate; The Maltose was freed from a small amount of higher oligosaccharides by recrystallisation (three times) from 80% ethanol.

Enzyme: Freshly harvested freeze dried cells (3·84 g.) were stirred with water (60 ml.) at room temperature for two hours. The suspension was then centrifuged at 5,000 revs. (2°C.) for 20 minutes. The resulting cell-free solution was removed.

Digest: The digest consisted of maltose (30 g.), 0·1 M- citrate buffer pH. 6·0 (150 ml.) and enzyme solution (20 ml.); toluene was added and the flask containing the digest was stoppered and incubated at 35°C.

Samples were examined chromatographically at intervals; the results

were identical to those obtained from the small scale digest. The reaction was terminated after 10 days incubation by heating the digest at 100°C. for 20 minutes. The resultant precipitated protein was removed by filtration; chromatographic examination of the filtrate gave results which were identical to those obtained using the unheated digest.

Preparation of the Charcoal - Celite Column.

The charcoal - Celite column (159) was prepared as follows:- B.D.H. activated charcoal (750 g.) and Celite 545 (750 g.), mixed dry, were poured, in the form of a slurry (4,900 ml. water) into a glass column (160 cm. x 7·5 cm.) containing a pad of Celite (2") supported on a glass wool pad on top of a porous disc. The slurry was added 3 - 4" at a time under gentle suction. The column was then washed thoroughly with water (20 l.). The rate of flow with water, which was run in from an aspirator placed 2 feet above the top of the column, was ca. 220 ml./hour.

(a) Fractionation of the Products of Enzyme Action.

The filtered digest was run into the column and mercuric iodide (ca. 500 m.g.) was added to ensure aseptic conditions. A step-wise elution of the component sugars was effected with water and with increasing concentrations of aqueous alcohol. Additional mercuric iodide was added at intervals. The eluates (1 - 2 l. portions), which were all found to be neutral, were evaporated to small volume (ca. 5 ml.) at 40°C. under reduced pressure; traces of charcoal and Celite were removed and the sugars examined chromatographically (Solvent 1, spray 1). Fractions containing like components were combined and the sugars isolated in crystalline form or, in the majority of cases, where crystallisation could not be induced, as freeze dried powders. Table 31 gives the nature and volume of eluant

TABLE 31

Eluant used	Vol. of eluant (litres)	R.g. values of component sugars	Yield (m.g.)	Fraction No.
Water	20·8	1·0	4800[x]	1
"	12·5	0·47	409	2
"	32·7	0·62, 0·47	2600	
"	38	0·62	2000	3
Ethanol (2%)	12	0·62	700	3
" (4%)	14	0·62	270	3
" (6%)	14·5	0·62 + mixture	390	
" (6%)	28	0·28	2940	4
" (6%)	85	0·41	3230	5
" (8%)	15	0·41 + mixture	60	
" (8%)	17·5	0·41, 0·31, 0·15	147	
" (8%)	12·5	0·31, 0·15	149	
" (8%)	7·5	0·15 + mixture	90	
" (8%)	7·5	0·15 + mixture (t)	65	
" (8%)	7·5	0·22 + 0·18	40	
" (8%)	59	0·18	549	6

x a further fraction of glucose, eluted from the column was accidentally lost before the yield could be determined.

used, and the R.g. values and approximate yields of the fractions; the numbered fractions are those which were subsequently characterised.

(b) Characterisation of Oligosaccharides.

Paper Partition Chromatography

The R.g. values were obtained using solvent 1 and spray 1.

R.g. values of the fractions

Fraction No.:	1	2	3	4	5	6
R.g. value :	1·0	0·47	0·62	0·28	0·41	0·18

R.g. values of reference sugars

Sugar	Glucose	Isomaltose	Maltose	Panose	Maltotriose
R.g. value	1·0	0·47	0.62	0·28	0·41

The authentic samples of maltotriose and panose were prepared and characterised by Dr. W. A. M. Duncan (137).

Estimation of Degree of Polymerisation

The sugars (ca. 5 m.g.) were dissolved in water (10 ml.) and the reducing power of samples (2 ml.) estimated using the Somogyi reagent; fractions 1 and 2 were heated for 15 minutes and the other sugars for 60 minutes. The reducing powers were calculated as equivalents of maltose. Samples (2 ml.) were hydrolysed with sulphuric acid (0·12 ml., 36 N.) at 100°C. for 2 hours, neutralised with sodium hydroxide using phenolphthalein as indicator, diluted to 5 ml. and the glucose estimated using Somogyi reagent. The degree of polymerisation were calculated as follows:-

$$\text{Weight of oligosaccharide/2 ml.} = \frac{\text{weight of glucose in hydrolysate} \times 162}{180}$$

$$\text{Degree of polymerisation} = \frac{\text{weight of oligosaccharide} \times 342}{\text{equivalent weight of maltose}}$$

Results.

Fraction:	1	2	3	4	5	6
Degree of polymerisation:	1·00	1·90	2·10	3·20	2·94	3·70

Controlled Overoxidation

The fractions (3 - 6 m.g.) were oxidised with sodium metaperiodate solution buffered at pH. 8 and the formaldehyde release determined (Method 5). On the basis that fraction 1 is a monosaccharide, fractions 2 and 3 are disaccharides, fractions 4 and 5 are trisaccharides and fraction 6 is a tetrasaccharide, the formaldehyde release was calculated in moles per mole of sugar.

Fraction:	1	2	3	4	5	6
Formaldehyde release:	1·01	0·08	1·70	0·94	2·80	1·98

Characterisation of fraction 1 as glucose.

This fraction, which had the same chromatographic mobility as glucose, had the same R.P. value and specific rotation. Overoxidation with sodium metaperiodate yielded the theoretical 1 mole of formaldehyde per mole of sugar.

Characterisation of fraction 2 as isomaltose.

This fraction had the same chromatographic mobility as isomaltose and had a D.P. of 2. On overoxidation with periodate no formaldehyde was liberated.

Partial acid hydrolysis. Fraction 2 (1 ml., 1%) and sulphuric acid (0·6 ml., 0·5N) were heated at 100°C. for 1 hour, neutralised and concentrated. Chromatographic examination (Solvent 1, spray 1) showed that fraction 2 and

glucose were the only reducing sugars present.

Characterisation of fraction 3 as maltose.

This fraction, which had the same chromatographic mobility as an authentic sample of maltose, had the same R.P. value and specific rotation. On partial acid hydrolysis, as above, glucose and unchanged fraction 3 were the only reducing sugars present. Overoxidation with periodate yielded two moles of formaldehyde per mole of disaccharide.

Characterisation of fraction 4 as panose.

This fraction, which had the same chromatographic mobility as an authentic sample of panose, had the reducing power of a trisaccharide. The specific rotation ($[\alpha]_D$, + 147·5, C. 2·3 in water) was similar to the values previously reported for panose, $[\alpha]_D$, + 148 (137); $[\alpha]_D$, + 150° (132) . Chromatographic analysis of a partial acid hydrolysate showed the presence of unchanged fraction 3, isomaltose, maltose and glucose.

Oxidation followed by partial acid hydrolysis (160).

Fraction 3 (5 ml., 1%), iodine (30 m.g.) and potassium hydroxide (0·2 ml., 1·0 M) were shaken together and allowed to stand for 30 minutes before the addition of a further portion of alkali (0·1 ml.). After standing overnight, a sample (1 ml.) was partially hydrolysed as before. Chromatographic analysis indicated the presence of isomaltose and glucose.

On overoxidation of fraction 4 with periodate, 0·94 mole of formaldehyde was released per mole of trisaccharide. The theoretical value for panose is 1·0.

Characterisation of fraction 5 as maltotriose.

This fraction had the same chromatographic mobility as an authentic

sample of maltotriose and had a reducing power corresponding to a trisaccharide. The specific rotation ($[\alpha]_D$, 161·1°, C., 2·7 in water) was similar to values previously reported for maltotriose $[\alpha]_D$ + 164° (137); $[\alpha]_D$ + 160° (161) Chromatographic examination of the partial acid hydrolysate showed the presence of unchanged fraction 5, maltose and glucose.

Fraction 5 (10 m.g.) was dissolved in a solution of unpurified salivary α - amylase (0·2 ml.), sodium chloride (4 m.g.) and 0·1 M- acetate buffer pH. 5·0 (0 2 ml.). Chromatographic examination after 24 hours incubation at room temperature showed the presence of fraction 5, maltose and glucose. A portion of fraction 5 was oxidised by alkaline hypoiodite as before. On partial acid hydrolysis, maltose and glucose were produced.

On overoxidation with periodate, 3 moles of formaldehyde were produced per mole of trisaccharide.

Characterisation of fraction 6 as 6^3 - α - glucosylmaltotriose.

This fraction, which had a reducing power equivalent to a tetrasaccharide, had R.g. value 0·18 (Solvent 1, spray 1). This R.g. value was similar to that previously reported for 6^3 - α - glucosylmaltotriose in the same solvent R.g., 0·17 (137) . The specific rotation ($[\alpha]_D$, + 172°, C., 0·4 in water) was slightly lower than the previously reported value $[\alpha]_D$, + 177° (137) Chromatographic examination of the partial acid hydrolysate showed the presence of fraction 6, panose, maltotriose, isomaltose, maltose and glucose. On partial acid hydrolysis of the material after oxidation with alkaline hypo-iodite, panose, isomaltose, maltose and glucose were produced. Overoxidation of fraction 6 with sodium periodate produced 2 moles of formaldehyde per mole of tetrasaccharide.

Transglucosylation with Maltose as Donor Substrate and Various Monosaccharides as Acceptor Molecules.

Digests were prepared containing maltose (100 m.g.) and each of D - xylose, D - lyxose, D - ribose, L - arabinose, D - galactose and D - fructose (100 m.g. in 2 ml. water) together with enzyme powder (20 m.g.). These digests were incubated at 35°C. and samples were examined at intervals by paper chromatography (Solvent 1, spray 2). After three to five days incubation, in addition to monosaccharides, maltose and oligosaccharides which had the same R.g. values and colour reaction with the spray reagent as isomaltose, maltotriose and panose, the digests showed the presence of new components as indicated in Table 32.

TABLE 32

Acceptor molecule	R.g. values of sugars in solvent 1.
D - xylose	0·64
D - lyxose	0·69
D - ribose	0·64
L - arabinose	0·72
D - galactose	0·32
D - fructose	-

The sugars synthesised as shown in Table 32 in digests containing pentoses all exhibited pink "spots" with spray reagent 2.

Transglucosylation with Various Donor Substrates.

Digests were prepared containing substrate (200 m.g., 1 ml.) and enzyme (20 m.g.). After 5 days incubation at 35°C., samples were examined chromatographically with results as shown in Table 33.

TABLE 33.

Substrate	R.g. values of sugars in solvent 1.
Isomaltose	1·0 (+); 0·47 (++); 0·19 (+); others (t).
Panose	1·0 (+); 0·62 (+); 0·47 (+); 0·28 (+); others (+)
Maltotriose	1·0 (+); 0·62 (+); 0·41 (+); others (+)
Methyl - - glucoside	1·0 (+), others (t).
Glucose	No synthesis
Xylose	No synthesis
Glucose - 1 - phosphate	No synthesis
Cellobiose	1·0 (+); 0·53 (++); 0·31 (t)
Sucrose	No synthesis.

The cellobiose digest, after incubation for a further 6 days, contained sugars having R.g. values:-

1·0 (+); 0·53 (++); 0·31 (+); 0·26 (t); 0·23 (t).

R.g. values of reference sugars were:- cellobiose, 0·53; cellotriose, 0·25; gentiobiose, 0·40; gentiotriose, 0·15.

SUMMARY.

The structural analysis of several protozoal polysaccharides has been carried out by chemical and enzymic methods, and a transglucosylase system in the ciliate Tetrahymena pyriformis has been investigated.

Since the majority of protozoal polysaccharides previously examined are branched α - 1:4 - glucosans, methods for the semi-micro analysis of such polysaccharides have been examined.

Determination of average chain length by periodate oxidation by the procedure of Potter and Hassid (J. Amer, Chem. Soc., (1948) p. 3488) was found to be unsatisfactory. The reaction conditions have therefore been modified. The periodate oxidation of maltose has been investigated.

The absorption spectra of the iodine complexes of branched α - 1:4 - glucosans have been measured in the presence and absence of ammonium sulphate. In aqueous solution the iodine complexes of glycogens and amylopectins have distinct spectra but in ammonium sulphate solution the spectrum is related to the degree of branching in the polysaccharide.

In the periodate oxidation and iodine absorption spectra experiments, polysaccharides from the following protozoa were used:- Tetrahymena pyriformis, Trichomonas foetus, Trichomonas gallinae and the holotrich ciliates of sheeps rumen.

A reserve polysaccharide extracted from Chilomonas paramecium was found to be of the starch type. The amylose content (45%) was higher than that of most plant starches (20 - 30%). The material was fractionated

into amylose and amylopectin components; the latter was similar to typical plant amylopectins. The amylose component, however, had a lower iodine affinity, blue value, and degree of polymerisation than most plant amyloses.

A second polysaccharide, prepared from Ochromonas malhamensis was found to be heterogeneous, the constituent sugars being glucose, galactose and mannose. A fraction containing glucose (85 %) and mannose (10%) was subjected to periodate oxidation and to enzymic and acid hydrolysis. This polysaccharide contained a large proportion of β - 1:3 - glucosidic linkages and had a degree of polymerisation in the range 30 - 60. The presence of a second type of linkage, as yet unidentified, was shown.

The transglycosylase activity of cell-free extracts of Tetrahymena pyriformis was examined. The following sugars were formed on incubation of such extracts with maltose:- glucose, maltose, isomaltose, maltotriose, panose, 6^3 - α - glucosylmaltoriose. Various monosaccharides could also act as acceptors for the transfer of α- glucosyl residues. In addition, the extract could catalyse trans - β- glucosylation using cellobiose as the donor substrate.

BIBLIOGRAPHY

(1) Haeckel, E., (1868). Jena. Z. Naturw., 4.

(2) Hutner, S. H., and Provasoli, L., (1951). Protozoa, Vol. 1, Acad. Press. Inc., New York.

(3) Lwoff, A., (1951). Protozoa, Vol. 1, Acad. Press. Inc., New York.

(4) Wenyon, C. M., (1926). Protozoology, Lond. Sch. Hyg. Trop. Med.

(5) Kudo, R. R., (1954). Protozoology, Charles C. Thomas, Publisher, Illinois.

(6) Catalogue of Protozoa (1958). J. Prot., 5, 1.

(7) Craig, C. F., and Faust, E. C., (1951). Clinical Parasitology, Henry Kimpton, Publisher, London.

(8) Brèchot, P., (1937). Compt. rend. soc. biol., 126, 555.

(9) Hutcheons, N. L., Podolsky, B., and Morales, M. F., (1948). J. Cell. Comp. Physiol., 32, 117.

(10) Bourne, E. J., Stacey, M., and Wilkinson, I. A., (1950). J. Chem. Soc., 2694.

(11) Lwoff, A., Ionesco, H., and Gutman, A., (1950). Biochim. et Biophys. Acta, 4, 270.

(12) Ruben, S., (1943), J. Amer. Chem. Soc., 65, 279.

(13) Oxford, A. E., (1951). J. Gen. Microbiol., 5, 83.

(14) Heald, P. J., and Oxford, A. E., (1953). Biochem. J., 53, 506.

(15) Manners, D. J., and Ryley, J. F., (1952). Biochem. J., 52, 480.

(16) Ryley, J. F., (1952). Biochem. J., 52, 483.

(17) Manners, D. J., and Ryley, J. F., (1955). Biochem. J., 59, 369.

(18) Forsyth, G., and Hirst, E. L., (1953). J. Chem. Soc., 2030.

(19) Mould, D. L., and Thomas, G. L., (1958). Biochem. J., 69, 327.

(20) Forsyth, G., and Hirst, E. L., (1953). J. Chem. Soc., 2132.

(21) Trevelyan, W. E., Procter, D. P., and Harrison, J. S., (1950). Nature, Lond., 166, 444.

(22) Partridge, S. M., (1949). Symp. Biochem. Soc., 3, 57.

(23) Rees, W. R., and Reynolds, T., (1958). Nature, Lond., 181, 767.

(24) Cifonelli, J. A., and Smith, F., (1954). Analyt. Chem., 26, 1132.

(25) Somogyi, M., (1952). J. Biol. Chem., 195, 19.

(26) Robinson, H. W., and Hogden, C. G., (1940). J. Biol. Chem., 135, 727.

(27) Chibnall, E. C., Rees, M. W., and Williams, E. F., (1943). Biochem. J., 37, 354.

(28) Fleury, P., and Lange, J., (1933). J. Pharm. Chim., 17, 107.

(29) Bricker, C. E., and Johnson, H. R., (1945). Analyt. Chem., 17, 400.

(30) Anderson, F. B., (1958). Ph.D. Thesis, University of Edinburgh.

(31) Hough, L., Powell, D. B., and Woods, B. M., (1956). J. Chem. Soc., 4799.

(32) Romjin, G., (1897). Z. Anal. Chem., 36, 18.

(33) Malaprade, L., (1928). Compt. rend., 186, 382.

(34) Jackson, E. L., (1944). Organic Reactions, Vol. II, 341.

(35) Bobbit, J. M., (1956). Adv. Carbohydrate Chem., Vol. XI, 1.

(36) Dyer, J. R., (1956). Methods of Biochem. Analysis, Vol. 3, 111.

(37) Jackson, E. L., and Hudson, C. S., (1937). J. Amer. Chem. Soc., 59, 994.

(38) Davidson, G. F., (1941). J. Textile Inst., 32, T., 109.

(39) Halsall, T. G., Hirst, E. L., and Jones, J. K. N., (1947). J. Chem. Soc., 1427.

(40) Halsall, T. G., Hirst, E. L., and Jones, J. K. N., (1947), J. Chem. Soc., 1399.

(41) Brown, F., Halsall, T. G., Hirst, E. L., and Jones, J. K. N., (1948). J. Chem. Soc., 27.

(42) Bell, D. J., and Manners, D. J., (1952), J. Chem. Soc., 3641.

(43) Anderson, D. M. W., Greenwood, C. T., and Hirst, E. L., (1955). J. Chem. Soc., 225.

(44) Manners, D. J., (1957). Adv. Carbohydrate Chem., Vol. XII, 261.

(45) Potter, A. L., and Hassid, W. Z., (1948). J. Amer. Chem. Soc., 70, 3488.

(46) Cori, G. T., and Larner, J., (1951). J. Biol. Chem., 188, 17.

(47) Schlamowitz, M., (1951). J. Biol. Chem., 188, 145.

(48) Polglase, W. J., Smith, E. L., and Tyler, F. H., (1952). J. Biol. Chem., 199, 97.

(49) Meyer, K. H., and Rathgeb, P., (1949). Helv. Chim. Acta, 32, 1102.

(50) Abdel-Akher, M., and Smith, F., (1951). J. Amer. Chem. Soc., 73, 994.

(51) Head, F. S. H., (1950). Nature, Lond., 165, 236.

(52) Head, F. S. H., and Hughes, G., (1952). J. Chem. Soc., 2046.

(53) Head, F. S. H., (1953). J. Textile Inst., 44, T., 209.

(54) Sarkar, P. B., (1951). Nature, Lond., 168, 122.

(55) Meyer, K. H., and Rathgeb, P., (1948). Helv. Chim. Acta, 31, 1540.

(56) Head, F. S. H., and Hughes, G., (1954). J. Chem. Soc., 603.

(57) Manners, D. J. in Manners, D. J., and Archibald, A. R., (1957). J. Chem. Soc., 2205.

(58) Manners, D. J., and Wright, A., unpublished.

(59) Carlqvist, B., (1948). Acta Chem. Scand., 2, 770.

(60) Manners, D. J., (1952). Ph.D. Thesis, Cambridge.

(61) Anderson, D. M. W., and Greenwood, C. T., (1955). J. Chem. Soc., 3016.

(62) Peat, S., Whelan, W. J., and Bailey, J. M., (1953). J. Chem. Soc., 1422.

(63) Larner, J., (1955). Methods in Enzymology, Vol. I, Acad. Press, New York, p. 222.

(64) Peat, S., Whelan, W. J., Hobson, P. N., and Thomas, G. J., (1954). J. Chem. Soc., 4440.

(65) Manners, D. J., and Khin Maung, (1956). Biochem. J., 63, 16 P.

(66) Swanson, M. A., (1948). J. Biol. Chem., 172, 825.

(67) Schlamowitz, M., (1951). J. Biol. Chem., 190, 519.

(68) Fairbrother, F., (1947). Nature, Lond., 160, 87.

(69) Fairbrother, F., (1948). J. Chem. Soc., 1051.

(70) Whelan, W. J., (1958). Encyclopedia of Plant Physiol., Vol. VI, p.154.

(71) Bottle, R. T., Gilbert, G. A., Greenwood, C. T., and Saad, K. N., (1953). Chem. and Ind., 541.

(72) Bell, D. J., (1955). Modern Methods of Plant Analysis, Springer-Verlag, Vol. II, p. 21.

(73) Meyer, K. H., and Bernfield, P., (1940). Helv. Chim. Acta, 23, 875.

(74) Baum, H., and Gilbert, G. A., (1956). J. Colloid Sci., 11, 428.

(75) Greenwood, C. T., and Robertson, J. S. M., (1954). J. Chem. Soc., 3769.

(76) Manners, D. J., and Khin Maung, (1955). Chem. and Ind., 950.

(77) Manners, D. J., (1955). Quart. Rev. Chem. Soc., IX, 73.

(78) MacWilliam, I. C., and Percival, E. G. V., (1951). J. Chem. Soc., 2259.

(79) Greenwood, C. T., and Das Gupta, P. C., (1958). J. Chem. Soc., 707.

(80) Arbuckle, A. W., (1958). Ph.D. Thesis, University of Edinburgh.

(81) Cowie, J. M. G., and Greenwood, C. T., (1957). J. Chem. Soc., 2862.

(82) Fleming, I. D., (1957). Ph.D. Thesis, University of Edinburgh.

(83) Deatherage, W. L., MacMasters, M. M., and Rist, C. E., (1955). Trans. Amer. Assoc. Cereal Chem., XIII, 31.

(84) Barker, S. A., Bourne, E. J., Peat, S., and Wilkinson, I. A., (1950). J. Chem. Soc., 3022.

(85) Pirt, S. J. and Whelan, W. J., (1951). J. Sci. Food Agric., 2, 224.

(86) Hobson, P. N., Whelan, W. J., and Peat, S., (1950). J. Chem. Soc., 3566.

(87) Khin Maung, (1956). Ph.D. Thesis, University of Edinburgh.

(88) Peat, S., Pirt, S. J., and Whelan, W. J., (1952). J. Chem. Soc., 714.

(89) Reazin, G. H., (1954). Amer. J. Bot., 41, 771.

(90) Ford, J. E., (1953). Brit. J. Nutrition, 7, 299.

(100) Hutner, S. H., Baker, H., Aaronson, S., Nathan, A., Rodriguez, E., Lockwood, S., Marvin, S., and Petersen, R. A., (1957). J. Protozool., 4, 259.

(101) Barker, S. A., Stacey, M., and Zweifel, G., (1957). Chem. and Ind., 330.

(102) Duncan, W. A. M., (1956). Ph.D. Thesis, University of Edinburgh.

(103) Anderson, F. B., (1958). Ph.D. Thesis, University of Edinburgh.

(104) Keilin, D., and Hartree, E. F., (1948). Biochem. J., 42, 221.

(105) Hough, L., and Perry, M. B., (1956). Chem. and Ind., 768.

(106) Carson, J. F., and Maclay, W. D., (1946). J. Amer. Chem. Soc., 68, 1015.

(107) Beicher, R., and Godbert, A. L., (1945). Semi-micro Quant. Org. Analysis, Longmans, p. 123.

(108) Anderson, F. B., Hirst, E. L., Manners, D. J., and Ross, A. G., (1958). J. Chem. Soc., in press.

(109) Bell, D. J., and Northcote, D. H., (1950). J. Chem. Soc., 1944.

(110) Black, W. A. P. (1948). Chem. and Ind., pp. 165, 169, 172.

(111) Aspinall, G. O., and Kessler, G., (1957). Chem. and Ind., 1296.

(112) Warsi, S. A. and Whelan, W. J., (1957). Chem. and Ind., 1573.

(113) Peat, S., Whelan, W. J., and Lawley, H. G., (1958). J. Chem. Soc., 724.

(114) Lwoff, A., (1923). Compt. rend., 176, 928-930.

(115) Seaman, G. R., (1952). Proc. Soc. Exptl. Biol. Med., 79 158.

(116) Slater, J. V., (1952). Science, 115, 376.

(117) Corliss, J. O., (1954). J. Protozoology, 1, 56.

(118) Hutner, S. H., and Trager, W., (1953). Ann. N. Y. Acad. Sci., 56, 815.

(119) Jirovec, O., (1950). Pathol. u. Bacteriol., 13, 129.

(120) Kidder, G. W., (1951). Ann. Rev. Microbiol., 5, 139.

(121) Kidder, G. W., and Dewey, V. C., (1945), Physiol. Zool., 18, 136.

(122) Miwa, T., and Takano, K., (1950). J. Biochem., Tokyo, 37, 435.

(123) Miwa, T., and Takano, K., (1950). Symp. enzyme chem. (Japan). 4, 76.

(124) Rabaté, M. J., (1935), Bull. Soc. Chim. Biol., Paris, 17, 572.

(125) Cohn, M., (1949). J. Biol. Chem., 180, 771.

(126) Koshland, D. E., and Stein, S. S., (1954). J. Biol. Chem., 208, 139.

(127) Bunton, C. A., Lewis, T. A., Llewllyn, D. R., Tristram, H., and Vernon, C. A., (1954). Nature, Lond., 174, 560.

(128) Wheland, G. W., (1950). Advanced Organic Chemistry ed. Wiley, New York.

(129) Ingold, C. K., (1953). Structure and Mechanism in Organic Chemistry, London, G. Bell and Sons, Ltd.

(130) Barker, S. A., and Bourne, E. J., (1953). Quart. Rev. Chem. Soc., Lond., 7, 56.

(131) Koshland, D. E., (1953). Biol. Rev., 28, 416.

(132) Pazur, J. H., and French, D., (1952). J. Biol. Chem., 196, 265.

(133) Koshland, D. E., (1954). Mechanism of Enzyme Action, p. 608, Baltimore, John Hopkins University Press.

(134) Pan, S. L., Andreasen, A. A., and Kolachov, P., (1950). Science, 112, 115.

(135) Barker, S. A., and Bourne, E. J., (1952). J. Chem. Soc., 209.

(136) Saroja, K., Ventkatarman, R., and Giri, K. V., (1955). Biochem. J., 60, 399.

(137) Duncan, W. A. M., and Manners, D. J., (1958), Biochem. J., 69, 343.

(138) Barker, S. A., and Carrington, T. R., (1953). J. Chem. Soc., 3588.

(139) Giri, K. V., Nagabhushanam, A., Nigam, V. N., and Belavadi, B., (1955). Science, 121, 898.

(140) Killey, M., Dimler, R. G., and Cluskey, J. E., (1955). J. Amer. Chem. Soc., 77, 3315.

(141) Koepsell, H. J., Tsuchiya, H. M., Hellman, N. N., Kazenko, A., Hoffman, C. A., Sharpe, E. S., and Jackson, R. W., (1953). J. Biol. Chem., 200, 793.

(142) Albon, N., Bell, D. J., Blanchard, P. H., Gross, D., and Rundell, J. T., (1953). J. Chem. Soc., 24.

(143) Pazur, J. H., (1955). J. Biol. Chem., 216, 531.

(144) Crook, E. M., and Stone, B. A., (1953). Biochem. J., 55, 25.

(145) Barker, S. A., Bourne, E. J., Hewitt, G. C., and Stacey, M., (1955). J. Chem. Soc., 3734.

(146) Anderson, F. B., and Manners, D. J., (1959). Biochem. J. (in press).

(147) Stacey, M., (1957). Biochimia, 22, 241.

(148) Watkins, W. J., (1958). Nature, Lond., 181, 117.

(149) Bailey, J. M., Whelan, W. J., and Peat, S., (1950). J. Chem. Soc., 3692.

(150) French, D., and Wild, G. M., (1953). J. Amer. Chem. Soc., 75, 4990.

(151) Whelan, W. J., and Bailey, J. M., (1954). Biochem. J., 58, 560.

(152) Peat, S., Whelan, W. J., and Rees, W. R., (1953). Nature, Lond., 172, 158.

(153) Peat, S., Whelan, W. J., and Rees, W. R., (1956). J. Chem. Soc., 44.

(154) Peat, S., Whelan, W. J., and Kroll, G. W. F., (1956). J. Chem. Soc., 53.

(155) Peat, S., Whelan, W. J., and Hinson, K. A., (1952). Nature, Lond., 170, 1056.

(156) Peat, S., Whelan, W. J., and Hinson, K. A., (1955). Chem. and Ind., 385.

(157) Gunja, Z., and Manners, D. J., unpublished.

(158) Buston, H. W., and Khan, A. H., (1956). Biochem. et Biophys. Acta, 19, 564.

(159) Whistler, R. L., and Durso, D. F., (1950). J. Amer. Chem. Soc., 72, 677.

(160) French, D., (1951). Science, 113, 352.

(161) Whelan, W. J., Bailey, J. M., and Roberts, P. J. P., (1953). J. Chem. Soc., 1293.

(162) Bourne, E. J., Haworth, N., Macey, A., and Peat, S., (1948). J. Chem. Soc., 924.

(163) Manners, D. J., and Thompson, J. L., unpublished.

The author wishes to thank Professor E. L. Hirst, C.B.E., F.R.S., for his interest in this work and to express his gratitude to Dr. D. J. Manners for his much appreciated advice and encouragement.

The author also wishes to thank Dr. D. J. Ryley for the provision of the protozoal polysaccharides and cultures, Dr. A. W. Arbuckle for potentiometric iodine titration of the starch fractions, and the Department of Scientific and Industrial Research for a Maintenance Allowance.

Preprinted from the Journal of the Chemical Society,
May, 1957, (423), pages 2205—2210.

423. α-1 : 4-*Glucosans. Part V.* End-group Assay of Glycogens by Periodate Oxidation, and the Oxidation of Maltose by Sodium Metaperiodate.*

By D. J. Manners and (in part) A. R. Archibald.

Fifteen samples of glycogen have been assayed by potassium metaperiodate oxidation, and with only two exceptions, average chain lengths of 10—14 glucose residues were obtained.

Oxidation of glycogen and of maltose, dissolved in sodium chloride, by sodium metaperiodate for 25 hr. at 2° (Potter and Hassid [1]) has been studied. Under these conditions, oxidation is incomplete.

Periodate oxidations have been widely used in structural investigations of polysaccharides, in particular, of starches, glycogens, and dextrans. By estimating the formic acid produced during oxidation, the proportion of triol groups in the polysaccharide can be assessed and, in starches and glycogens, the ratio of non-terminal to non-reducing terminal glucose residues (*i.e.*, average chain length, $\overline{CL}$) determined.[2] Further, examination of an acid hydrolysate of a periodate-oxidised glucosan enables 1 : 2- or 1 : 3-glucosidic linkages to be detected.[3] In the present investigation, the average chain lengths of several samples of glycogen have been determined by (*a*) potassium metaperiodate oxidation at room temperature, and (*b*) sodium metaperiodate oxidation at 2°. A preliminary account of part of this work has been published.[4]

Table 1. *End-group assay of glycogens by oxidation with potassium periodate.*

Source of glycogen [a]	Method of isoln.[b]	Method of purifn.[c]	$[\alpha]_D$ (in H_2O)	$\overline{CL}$	Source of glycogen [a]	Method of isoln.[b]	Method of purifn.[c]	$[\alpha]_D$ (in H_2O)	$\overline{CL}$
Cat liver IV	P	A	—	13	Rabbit liver III...	W	A	+196°	13
Cat liver VI	P	A	—	12	Rabbit liver IV	W	A	—	13
Fœtal pig liver ...	W	A	+191°	11	Rabbit liver V ...	W	A	+196	12
Helix pomatia I ...	P	A	+192	10	Rabbit liver X ...	C	E	+193	12
Helix pomatia II	P	A	+182	7	*Tetrahymena pyriformis* II	P	A	+195	14
Mytilus edulis IV	W	PA	+196	12	*Trichomonas gallinae* II	P	A	+200	13
Mytilus edulis V...	W	A	—	9					
Mytilus edulis VI	C	A	+195	13					
Rabbit liver II ...	P	A	+198	12					

[a] Roman numerals refer to different samples from the same biological source. [b] P = Pflüger method; W = hot-water extraction; C = commercial preparations. [c] A = acetic acid precipitation (Bell and Young, *Biochem. J.*, 1934, **28**, 282); PA = deproteinisation with picric acid; E = electrodialysis.

Assay of glycogen by oxidation with potassium periodate was first made by Halsall, Hirst, and Jones,[5] $\overline{CL}$ values being calculated from the production of formic acid after 150 hours' oxidation. Later, Bell and Manners [6] found that samples of mammalian-muscle glycogen ($\overline{CL}$ 12, by methylation) had apparent chain lengths of 15—16 after 150 hours' oxidation. However, when oxidation was continued to maximum production of formic acid (after 300—400 hours' oxidation), $\overline{CL}$ values of 12 ± 1 were obtained. This apparent discrepancy was ascribed to variation in the oxidation rate with room temperature.[7] A similar effect was also noted by Carlquist,[8] who found that in 144 hr. at 15° or 21° a glycogen sample gave 8·4 or 9·5 moles of formic acid per 100 glucose residues. Accordingly, end-group assays have been carried out on 15 different samples of glycogen, and $\overline{CL}$ values, evaluated from the final constant concentrations of formic acid, are recorded in Table 1, together with the specific rotations and methods of preparation of the glycogens.

* Part IV, *J.*, 1956, 2831.

During isolation of glycogen by the Pflüger method (digestion of the tissues with hot 30% potassium hydroxide), there is no appreciable alkaline-degradation of the polysaccharide.[9] By methylation, the glycogens from rabbit liver III and X and *Helix pomatia* I had chain lengths of 12.[10-12] The latter type of glycogen therefore differs significantly in degree of branching from *Helix pomatia* II glycogen isolated in 1949; the presence of 7-unit chains in this has been confirmed. Fœtal pig liver glycogen, which has not previously been studied, resembles the majority of mammalian glycogens in degree of branching.

An alternative periodate method used by Potter and Hassid [1] involves oxidation at 2° of a solution of the polysaccharide in 1·5% sodium chloride with 1·5 mols. of sodium metaperiodate and determination of the formic acid produced after 25 hr. Chain lengths of 22—27 were reported for various amylopectins, although these results do not appear to have been confirmed by methylation assay of the *same* samples. Other end-group assays of glycogens and amylopectins by this method have been reported; [13-15] the $\overline{CL}$ values were greater than those obtained by other methods of assay of the same samples. In particular, the results differed from enzymic assays (using phosphorylase and amylo-1 : 6-glucosidase), as shown in Table 2.

TABLE 2. *Chain lengths of glycogens and amylopectins determined by periodate oxidation and enzymic methods.*

Sample	Sodium periodate oxidn.[a]	Enzymic assay	Ref.	Sample	Sodium periodate oxidn.[a]	Enzymic assay	Ref.
Rabbit liver glycogen	18	14·7	13	Potato amylopectin	27	21·8	13
Rabbit liver glycogen	22	15·9	16	Sago amylopectin ...	22	17	13
Rabbit liver glycogen	23	17·2	16	Wheat amylopectin...	23	18·5	13
Corn amylopectin ...	26	21·2	13				

[a] Procedure of Potter and Hassid.[1]

It appears that with Potter and Hassid's procedure periodate oxidation and, hence, production of formic acid, are incomplete after 25 hr.; it has therefore been applied to glycogens already assayed by potassium periodate. Six samples of glycogen (and one of amylopectin), in sodium chloride solution, were oxidised with sodium metaperiodate at 2°, and the concentration of formic acid was determined after 25 hr. (Table 3). Since the

TABLE 3. *End-group assays of glycogens by periodate oxidation.*

Sample	Potassium periodate oxidn.*	Sodium periodate oxidn.†		Sample	Potassium periodate oxidn.*	Sodium periodate oxidn.†	
		A	*B*			*A*	*B*
Ascaris lumbricoides	12	15—16	11	*Mytilus edulis* VI...	13	—	14
Cat liver VI	12	—	12	Rabbit liver I	13	—	13
Commercial ‡	—	—	12	Rabbit liver II ...	12	14	—
Helix pomatia II ...	7	9	—	Rabbit liver V ...	12	—	12
Human liver	6	8	—	Rabbit liver X.....	12	—	12
Human muscle ...	12	—	11	*Trichomonas fœtus*	15	18	15
Mytilus edulis V ...	9	13	—				

* See Table 1 and ref. 6. † *A*, Potter and Hassid's procedure; *B*, modified procedure.

‡ Purchased from British Drug Houses Ltd. [waxy maize starch had $\overline{CL}$ values of 18 and 22 by potassium and sodium periodate oxidation (method *A*) respectively].

$\overline{CL}$ values from the titres after 25 hr. were greater than those from potassium periodate assay, production of formic acid was incomplete. Six further quantitative experiments showed that only 80—90% of the theoretical periodate was reduced after 25 hr. Schlamowitz's report [14] that formic acid was completely liberated within 20—25 hr. could not be confirmed (see p.).

The period of 25 hr. for oxidation was chosen by Potter and Hassid [1] on the grounds that their " model " saccharide, maltose, yielded the theoretical 3 mols. of formic acid in this time. Their method is therefore based on the unproved assumption that oxidation of a disaccharide occurs at the same rate as that of a polysaccharide of molecular weight

$\sim 10^7$. We have noted, however, that different samples of glycogen are oxidised, under identical conditions, at slightly differing rates. We have re-examined the periodate oxidation of maltose in sodium chloride. In one experiment, after 25 hr. 2·3 mols. of formic acid were produced, and 4·7 mols. of periodate reduced. In additional experiments, maltose, in water or in 3% sodium chloride, was oxidised with varying amounts of sodium metaperiodate. After 25 hr. 1·7—2·6 mols. of formic acid were present, and after *ca.* 120 hr. 2·4—3·1 mols. Although strictly reproducible results could not be obtained in the presence of sodium chloride (see p.), release of formic acid and uptake of periodate were never theoretical after 25 hours' oxidation at 2°.

The formation and subsequent hydrolysis of a formyl ester were incidentally indicated, since production of the third mol. of formic acid continues in the *absence* of periodate. A mixture of maltose in sodium chloride and sodium metaperiodate was divided after 48 hr. at 2° when it contained 2·6 mols. of formic acid. One half was stored at 2° for 24 hr. : the formic acid content increased to 2·9 mols. To the other half ethylene glycol was added, to reduce the remaining periodate, and the formic acid determined immediately and at intervals thereafter. During 24 hr. the formic acid content increased slowly from 2·6 to 2·9 mols. In a similar oxidation by aqueous sodium metaperiodate, we found 2·4 mols. of formic acid released after 48 hours' oxidation, 2·7 mols. after neutralisation of periodate and storage at 2° for 24 hr., and 2·6 mols. after 72 hours' total oxidation. Aliphatic formyl esters are also slowly hydrolysed in presence of sodium metaperiodate at 2° without consumption of periodate. It is apparent that the periodate oxidation of maltose resembles that of lactose [17] and cellobiose,[18] and involves (*a*) an initial oxidation in which 4 mols. of periodate are reduced and 2 mols. of formic acid produced, and (*b*) a slower stage involving the release of a third mol. of formic acid by hydrolysis of a formyl ester.

The above results on the rate of production of formic acid have been confirmed by others. Morrison and his co-workers [19] found that maltose oxidised by Potter and Hassid's procedure gave 2·5 mols. of formic acid, whilst Potter and his collaborators [20] observed that at 3° the expected 3 mols. of formic acid were not produced after 9 days.

For the end-group assay of glycogens, Potter and Hassid's method has therefore been modified : oxidation in *aqueous* solution is continued for 7—10 days, and the maximum concentration of formic acid determined. With " model " compounds consisting of glycogens already assayed by potassium periodate, the oxidation (determined by periodate-uptake and formic acid production) was normally complete within 7 days. Typical results are reported in Table 3. We noted, however, that with certain mammalian liver glycogens formic acid is produced unexpectedly slowly; [21] the reasons for this are being investigated.

The present study provides further evidence that the *average* length of the chains in glycogens is normally *ca.* 12 glucose residues. We have now assayed some 30 samples of glycogen and, of these, 23 had $\overline{CL}$ values of 10—14. These results agree with those of Abdel-Akher and Smith [22] who for another 37 individual glycogens found average chain lengths of 10—14. This suggests that in most animal tissues the *relative* activity of phosphorylase and branching enzyme during glycogen synthesis is very similar. However, the activity of this enzyme system in different specimens of *Mytilus edulis* appears to vary since glycogens with $\overline{CL}$ values of *ca.* 5, 9, 12, 13, and 17 have been isolated (see Table 1 and ref. 6).

Experimental

Glycogen Samples.—We are indebted to Dr. J. S. D. Bacon for *Mytilus edulis* V glycogen, to Dr. G. D. Greville for the cat liver glycogens, to Dr. E. E. Percival for rabbit liver X glycogen and to Dr. J. F. Ryley for the protozoal glycogens (cf. ref. 23). *Helix pomatia* I glycogen was prepared by deacetylation of the corresponding acetate which was kindly provided by Dr. D. J. Bell. *Mytilus edulis* VI glycogen was purchased from L. Light and Co, Ltd. The remaining

samples were isolated and purified as indicated in Table 1. The rotations of 0·2—0·4% glycogen solutions were measured in 2 dm. polarimeter tubes.

Potassium Periodate Oxidation of Glycogens.—The method previously described [6] was used.

Sodium metaperiodate oxidations.

Analytical Methods.—Formic acid was determined, after neutralisation of periodate with ethylene glycol, by titration with 0·01N-sodium hydroxide in a stream of carbon dioxide-free air with (*a*) methyl-red as indicator, when repeating Potter and Hassid's experiments,[1] or (*b*) a glass electrode and pH meter to an end-point at pH 5·8. Periodate uptake was estimated by the methods of Barnebey [24] or Fleury and Lange.[25] Results are expressed as moles per mole of maltose or, for glycogens, moles per mole of anhydroglucose residue.

Oxidations in Presence of Sodium Chloride.—During oxidations of glycogen or maltose in 1·5% sodium chloride solution with sodium metaperiodate, reagent blanks were also analysed. On storage at 2°, these became acid (pH *ca.* 3), and periodate was precipitated. Production of formic acid is therefore calculated from sodium hydroxide titres after correction for the *initial* acidity of the reagent blanks. Further, the results are approximate since the sodium hydroxide titrations include not only release of formic acid by periodate oxidation, but also, and to an unknown extent, acidity due to the interaction of sodium chloride and metaperiodate. The precipitation of periodate, which occurred within 24—96 hr., prevented accurate measurement of periodate reduction.

(a) *Glycogens.* Glycogen (30—280 mg.) in 3% sodium chloride (5 ml.) was oxidised with 0·27—0·40M-sodium metaperiodate (5 ml.) at 2° for 25 hr. Ethylene glycol (neutral; 3 ml.) was added, and the mixture kept for 1 hr. at room temperature in the dark, before titration. A control of sodium chloride and metaperiodate was similarly analysed. The results are given in Table 3.

The rate of formic acid production was studied by treating rabbit liver IV glycogen (189·8 mg.) and *Mytilus edulis* I glycogen (110·0 mg.) in 3% sodium chloride (10 ml.) with 0·37M-sodium metaperiodate (10 ml.) at 2° :

Time of oxidn.	Apparent chain length (glucose residues)	
(hr.)	Rabbit liver IV glycogen	*Mytilus edulis* I glycogen
51	16·2	16·2
100	15·0	15·2
166	14·6	14·2
291	12·6	13·2

For measurement of periodate uptake, 50 mg. of glycogen were oxidised for 25 hr. The results were glycogen from *Ascaris lumbricoides*, 0·98 mole per anhydroglucose residue; cat liver IV, 0·89; cat liver VI, 0·94; horse muscle, 0·92; fœtal sheep liver, 0·95, and rabbit liver IV, 0·94. On complete oxidation, these glycogens reduce 1·08—1·09 mols. of periodate.

(b) *Maltose.* Maltose hydrate (223·4 mg.), dissolved in 3% sodium chloride (10 ml.), was oxidised with 0·37M-sodium metaperiodate (10 ml.; 6 mols.) at 2°. The results were :

Time of oxidn. (hr.)	2	4	25	48	72
Formic acid prodn. (mols.)	1·5	1·7	2·3	2·7	3·1

In a second experiment, anhydrous maltose (162·8 mg.) in 3% sodium chloride was treated with 0·27M-sodium metaperiodate (10 ml.; 6 mols.) at 2° :

Time of oxidn. (hr.)	0·5	1	2	22	25	96	145
Periodate uptake (mols.)	3·2	3·9	4·0	4·6	4·7	—	—
Formic acid prodn. (mols.)	1·1	1·2	1·4	2·3	2·3	2·7	3·3

Maltose was also oxidised with varying amounts of sodium metaperiodate (4·4—34·0 mols.); after 25 hr. 1·7—2·5 mols. of formic acid were released, and after 95 hr. 2·5—2·9 mols.

In the above experiments, free iodine was present after *ca.* 90 hr. showing that over-oxidation had occurred.

Oxidations in Absence of Sodium Chloride.—In these oxidations, the reagent control of aqueous sodium metaperiodate was stable on storage at 2°.

(a) *Maltose.* Maltose hydrate (220·4 mg.) in water (10 ml.) was oxidised with 0·37M-sodium metaperiodate (10 ml.; 6 mols.) at 2° with results as follows :

Time of oxidn. (hr.)	2	4	25	48	72
Periodate uptake (mols.)	3·6	—	4·2	4·2	4·4
Formic acid prodn. (mols.)	1·4	1·6	2·2	2·5	2·8

On repetition, 2·1 mols. of formic acid were produced, and 4·2 mols. of periodate consumed after 25 hr. Oxidation of maltose with 4·0 or 11·6 mols. of periodate gave the following results :

Time of oxidn. (hr.)	2	4	25	48	72
Formic acid prodn. (mols.) :					
(*a*) 4·0 mols. of oxidant	1·3	1·4	2·0	2·1	2·3
(*b*) 11·6 mols. of oxidant	1·8	1·9	2·6	2·9	3·0

In the above experiments, the " formic acid " production is slightly lower (*ca.* 0·2 mol. of apparent formic acid) than in those in presence of sodium chloride. The difference is attributed to the acidity developed during the interaction of sodium chloride and metaperiodate.

(b) *Glycogens. Trichomonas fœtus* glycogen [23] (106·4 mg.) in water (25 ml.) was oxidised with 0·37M-sodium metaperiodate (5 ml.) at 2°. Portions (5 ml.) were removed at intervals, ethylene glycol (1 ml.) was added, and the formic acid titrated, with the following results :

Time of oxidn. (days)	2	4	6	10
Formic acid prodn. (mg.)	1·66	1·81	1·93	2·00
Apparent chain length (glucose residues)	18·0	16·4	15·4	15·0

Under similar conditions, *Ascaris lumbricoides* glycogen (100 mg.) gave 2·26 mg. of formic acid after 3 days, and 2·35 mg. after 5 days; 0·99 and 1·05 mols. of periodate were reduced within 2 and 3 days, respectively.

For end-group assays, glycogen (*ca.* 100 mg.) in water (23 ml.) was oxidised with 0·4M-sodium metaperiodate (2 ml.) at 2°. Portions (5 ml.) were removed at intervals for determination of formic acid. $\overline{CL}$ values, calculated from the final formic acid concentration, are given in Table 3; 1·06—1·09 mols. of periodate were reduced during these oxidations.

Ascaris lumbricoides glycogen (102·5 mg.) and *Mytilus edulis* VI glycogen (95·8 mg.) were also oxidised at room temperature (15—17°). The final production of formic acid (after 8 days) was 2·54 and 2·13 mg. respectively, corresponding to average chain lengths of 11 and 13 glucose residues. During the oxidation of glycogens with a *limited* excess of periodate (*ca.* 30%), appreciable " over-oxidation " does not therefore occur.

Hydrolysis of Formyl Esters.—Mixtures of maltose (*a*) in sodium chloride and (*b*) in water with sodium metaperiodate were divided after 48 hours' oxidation at 2°. One-half of each solution was stored at 2° for a further 24 hr. Ethylene glycol was added to the remaining solutions, which were then titrated with sodium hydroxide (methyl-red). The neutralised solutions were stored at 2° for 24 hr., alkali being added at intervals to maintain the pH. Results were :

Time after redn. of periodate (hr.)	0	2	3	24
Formic acid prodn. (mols.) (*a*)	2·6	2·7	2·8	2·9
(*b*)	2·4	2·5	2·6	2·7

The normal oxidations resulted in the production of (*a*) 2·9 and (*b*) 2·6 mols. of formic acid after 24 hr.

At 2°, and in presence of 3% sodium chloride and 0·37M-sodium metaperiodate, ethyl and *n*-propyl formate were slowly hydrolysed. No periodate was consumed.

The authors are indebted to Professor E. L. Hirst, F.R.S., for his interest and encouragement, to Mr. L. M. Atherden for experimental assistance, and to the Department of Scientific and Industrial Research for a maintenance grant (to A. R. A.).

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF EDINBURGH. [*Received, December 19th,* 1956.]

[1] Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488.
[2] Brown, Dunstan, Halsall, Hirst, and Jones, *Nature*, 1945, **156**, 785.
[3] Hirst, Jones, and Roudier, *J.*, 1948, 1779.
[4] Manners, *Biochem. J.*, 1953, **55**, xx.
[5] Halsall, Hirst, and Jones, *J.*, 1947, 1399.
[6] Bell and Manners, *J.*, 1952, 3641.
[7] Manners, Ph.D. Thesis, Cambridge, 1952.
[8] Carlquist, *Acta Chem. Scand.*, 1948, **2**, 770.
[9] Greenwood and Manners, *Proc. Chem. Soc.*, 1957, 26.
[10] Bell, *Biochem. J.*, 1935, **29**, 2031.
[11] Haworth and Percival, *J.*, 1932, 2277.
[12] Baldwin and Bell, *Biochem. J.*, 1940, **34**, 139.

[13] Cori and Larner, *J. Biol. Chem.*, 1951, **188**, 17.
[14] Schlamowitz, *ibid.*, p. 145.
[15] Polglase, Smith, and Tyler, *ibid.*, 1952, **199**, 97.
[16] Illingworth, Larner, and Cori, *ibid.*, p. 631; Schlamowitz, personal communication.
[17] Meyer and Rathgeb, *Helv. Chim. Acta*, 1948, **31**, 1540.
[18] Head and Hughes, *J.*, 1954, 603.
[19] Morrison, Kuyper, and Orten, *J. Amer. Chem. Soc.*, 1953, **75**, 1502.
[20] Potter, Silveira, McCready, and Owens, *ibid.*, p. 1335; see also Wolff, Hofreiter, Watson, Deatherage, and MacMasters, *ibid.*, 1955, **77**, 1656.
[21] Cf. Perlin, *ibid.*, 1954, **76**, 4101.
[22] Abdel-Akher and Smith, *ibid.*, 1951, **73**, 994.
[23] Manners and Ryley, *Biochem. J.*, 1952, **52**, 480; 1955, **59**, 369.
[24] Barnebey, *J. Amer. Chem. Soc.*, 1916, **38**, 330.
[25] Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107.

PRINTED IN GREAT BRITAIN BY RICHARD CLAY AND COMPANY, LTD.,
BUNGAY, SUFFOLK.